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Investigating the Role of SUMOylation of Eukaryotic Translation Initiation Factors

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**A thesis submitted to the University of Sussex
for the degree of Doctor of Philosophy**

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Declaration

I hereby declare that this thesis has not been, and will not be, submitted in whole or in part to another University for the award of any degree. The work described herein is my own work except where otherwise stated.

Jirapas Jongjitwimol

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Abstract

Dysregulation of translation has a direct effect on growth control in mammalian cells. The initiation step of translation is an important point of control of gene expression and a rate-limiting step for protein synthesis. Initiation requires initiation factors (eIFs) that are important for the activation of both mRNA and the recruitment of ribosomal subunits. Previously, post-translational modifications (PTMs), such as phosphorylation, have been shown to be an important part in fine-tuning translation. SUMOylation is another PTM that affects a number of fundamental cellular processes, including the response to DNA damage, metabolic regulation and protein trafficking. Many eIFs have been identified in proteomic screens as SUMOylated targets, but to date most of these modifications have not been confirmed. Preliminary work from the Watts lab indicated that some eIFs co-purified with the *S. pombe* SUMO protease Ulp2. The aim of my project was to determine whether components of the eIF4^F complex are SUMOylated and to initiate studies to investigate the role of this modification. The first results chapter investigates eIF4G, eIF3h and Sla1 (the La protein homologue) and demonstrates that eIF4G and Sla1, but not eIF3h, are SUMOylated in *S. pombe*. These experiments were then extended to mammalian cells. The effects of stress conditions on protein synthesis and SUMOylation in a range of cell lines were first analysed. SUMO localisation was altered in response to sodium arsenite (AR) and ionising radiation (IR). In most cell types tested, for example, after IR treatment, SUMO1 went to nuclear foci in HeLa cells, but was more abundant in the cytoplasm following exposure to AR. Next, *in vivo* and *in vitro* SUMOylation assays were used to demonstrate that mammalian eIF4G and eIF4A are both SUMOylated. Mass spectrometric analysis identified the SUMOylation sites in eIF4G, as K1386 and K1588. Those of eIF4AI and eIF4AII are K225 and K226, respectively. Mutated eIF4AII was introduced into cells to investigate the role of SUMOylation of this factor. Colocalisation of eIF4A/eIF4G and SUMO1 shows that, in AR-treated cells, SUMO1 colocalises with eIF4A and eIF4G in the cytoplasmic stress granules, especially at their edges. In contrast, in IR-treated cells, the colocalisation of eIF4G/eIF4A with SUMO1 is much more in the nucleus, compared to that in untreated cells, suggesting that eIF4G/eIF4A and SUMO1 may have a cellular role in some aspects in response to AR and IR.

Abbreviations

4E-BP	eIF4E binding protein
ABP1	actin-binding protein
Ade	adenine
AGO, Ago	Argonaute protein
AKT	protein kinase B (PKB)
Amp	ampicillin
AP	apurinic/apyrimidinic site, abasic site
APE	AP endonuclease
APL	acute promyelocytic leukaemia
APS	ammonium persulphate
AR	sodium arsenite
ATM	ataxia telangiectasia mutated
BER	base-excision DNA repair
bp	base pairs
BSA	bovine serum albumin
CAF1	CCR4-associated factor 1
CBP	cAMP response element-binding protein (CREB)-binding protein
CCR4	carbon catabolite repressor protein 4
cDNA	complementary DNA
Chx	cycloheximide
CIP	calf intestine alkaline phosphatase
DAPI	4', 6-diamidino-2-phenylindole
Daxx	death-domain associated protein
Dbp2	DEAD-box protein 2 (ATP-dependent RNA helicase)
DCP	decapping protein
DDX	DEAD-box protein
Dhp1	Dhm1-like protein (5'-3' exoribonuclease)
DMEM	Dulbecco's modified eagle medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dNTP	deoxynucleoside triphosphate

DTT	dithiothreitol
DUB	deubiquitylating enzyme
ECL	enhanced chemiluminescence
EDC	enhancer of decapping protein
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eIF	eukaryotic initiation factor
ELN	extra Low nitrogen
EMM	Edinburgh minimal medium
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Exo2	exonuclease 2
FAG	fragment of eIF4G generated by apoptosis
FCS	foetal calf serum
GCN2	general control nonderepressible 2
Gpd1	glyceraldehyde-3-phosphate dehydrogenase 1
Gpd3	glyceraldehyde 3-phosphate dehydrogenase 3
GW182	glycine-tryptophan protein of 182 kDa
Gy	Gray
HA	hemagglutinin
HRI	heme-regulated inhibitor kinase
Hsc1/Sks2	heat shock cognate protein 1
IF	immunofluorescence
IMS	industrial methylated spirits
IPTG	isopropyl β -D-1-thiogalactopyranoside
IR	ionising radiation
IRE	iron regulatory element
IRES	internal ribosome entry site
IRP	IRE-binding protein
KAN, kan	kanamycin
LB	lysogeny broth
Leu	leucine
LiAc	lithium acetate

m ⁷ GpppN	7-methylguanosine-triphospho-5'-ribonucleoside
m ⁷ GTP	7-methyl guanosine triphosphate
MAPK	mitogen-activated protein kinase
Met-tRNA _i	initiator methionyl tRNA
miRISC	miRNA-induced silencing complex
miRNA	microRNA
Mnk	MAP-kinase interacting kinase
MOI	multiplicity of infection
MOPS	3-morpholinopropane-1-sulfonic acid
MS	mass spectrometry
mTOR	mammalian target of rapamycin
NAT	nourseothricin sulfate
Noc2	nucleolar complex 2 protein
Nop2	nucleolar protein 2 (RNA methyltransferase)
NOT1	negative regulator of transcription subunit 1
NT	non-transfected
OD	optical density
ORF	open reading frame
p53	tumor suppressor p53
PABP	poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
P-body	processing body
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween-20
PCNA	proliferating cell nuclear antigen
PD	pulled down, as in affinity purification using Ni ²⁺ agarose beads
PEG	polyethylene glycol
PERK	PKR-like ER-localized eIF2 α kinase
Pfk1	6-phosphofructokinase 1
PI3K	phosphatidylinositol 3-kinases
PIC	pre-initiation complex
PKC α	protein kinase C α
PKR	protein kinase R

PML	promyelocytic leukaemia protein
PMSF	phenylmethanesulphonyl fluoride
Prp19	pre-mRNA processing factor 19
PTM	post-translational modification
PVDF	polyvinylidene difluoride
RanGAP	Ran GTPase activating protein
Rfc5	DNA replication factor C complex subunit 5
RISC	RNA-induced silencing complex
RMCE	recombination-mediated cassette exchange
Rml2	mitochondrial ribosomal protein subunit L2
Rpa1, Rpa2	60S acidic ribosomal protein A1, A2
Rpl301	60S ribosomal protein L3
Rpl302	60S ribosomal protein L3
rpm	revolutions per minute
RRM	RNA recognition motif
Rrp5	60S acidic ribosomal protein P1-alpha 5
SAE	SUMO-activating enzyme
SDS	sodium dodecyl sulphate
SENP	SUMO/sentrin specific peptidase
SG	stress granule
Sla1	synthetic lethal with ABP1
Sp100	Sp100 nuclear antigen
STUbL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin-like molecule
TCA	trichloroacetic acid
Tcg1	single-stranded telomeric binding protein Tgc1
TDG	thymine-DNA glycosylase
TE	Tris-EDTA
TEMED	tetramethylethylenediamine
TEV	tobacco etch virus protease
TFA	trifluoroacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
Ulp1, Ulp2	ubiquitin-like-specific protease 1, 2

Upf1	up frameshift mutation 1 (ATP-dependent RNA helicase)
Ura	uracil
UT	untreated
UTR	untranslated region
WB	western blotting
WCE	whole cell extract
WT, wt	wild-type
X-gal	5-bromo-4-chloro-indolyl- β -D-galactopyranoside
YE	yeast extract
YNB	yeast nitrogen base
YNBA	yeast nitrogen base agar

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Chapter 1

Introduction

This work is a study on the SUMO modification of eukaryotic translation initiation factors (eIFs) from both the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) and humans. This chapter will provide some information involving protein synthesis, in particular translation initiation and translational regulation. This chapter also includes post-translational modification, specifically SUMOylation. In addition, previous studies from the Watts lab will be described as preliminary results have demonstrated a number of eIFs to be potential SUMO targets.

1.1 Protein synthesis

Protein synthesis is the cellular process that produces new proteins. Eukaryotic transcription occurs in the nucleus and converts the information in DNA into messenger RNA (mRNA) by DNA-directed RNA polymerase II. Pre-mRNA is capped by 7-methyl guanosine triphosphate (m⁷GTP), spliced and then polyadenylated in the nucleus before being translocated to the cytoplasm. During translation, spliced mRNA associates with ribosomes and transfer RNA (tRNA) in order to generate proteins (Sonnenberg and Hinnebusch, 2009; Jackson *et al.*, 2010). Following protein synthesis, post-translational modification (PTM) can occur; these are mostly reversible and modulate the function of proteins. Thus, PTMs have important roles in biological processes. There are several types of PTMs which depend on a characteristic feature of proteins such as ubiquitylation, ubiquitin-like modification, acetylation, methylation, phosphorylation and glycosylation (Walsh *et al.*, 2005; Prabakaran *et al.*, 2012).

Regulation of gene expression or protein synthesis occurs at multiple steps, for example, from the process of transcription initiation to PTM of the polypeptide chain. Translational regulation is widely used to control gene expression: there are two main mechanisms, namely global regulation and mRNA-specific regulation (Gebauer and Hentze, 2004; Preiss, 2005; Le Quesne *et al.*, 2010). Dysregulation of this process may cause a range of diseases such as diabetes, neurological disease and some cancers (Gingras *et al.*, 1999; Hollams *et al.*, 2002; Le Quesne *et al.*, 2010)

1.2 Basic mechanism of eukaryotic translation initiation

Translation can be divided into three main stages: initiation, elongation and termination. The initiation stage is understood to be the main point of control of translation (Jackson *et al.*, 2010). Translation initiation is a process in which an initiator tRNA-ribosome complex is formed and recruited to the start codon (usually AUG) on mRNA (Figure 1.1). The formation of the eukaryotic translation initiation apparatus requires a large number of proteins termed eukaryotic initiation translation factors (eIFs) (Sonenberg and Hinnebusch, 2009; Jackson *et al.*, 2010). Eukaryotic translation is initiated by a Met-tRNA, known as initiator Met-tRNA (Met-tRNA_i), which interacts specifically with an eIF2-GTP complex, also known as eIF2 ternary complex (Jackson *et al.*, 2010). eIF3, eIF1, eIF1A and eIF5 are recruited to the small (40S) ribosomal subunit (Hinnebusch, 2006). Subsequently, the eIF2 ternary complex attaches to the ribosomal P site of 40S ribosomal subunits, bound to eIF3, eIF1, eIF1A and eIF5, to form a 43S pre-initiation complexes (PIC) (Pestova *et al.*, 2001; Hinnebusch, 2006; Jackson *et al.*, 2010).

To load 43S PIC onto mRNA requires the collective action of eIF4F and eIF4B or eIF4H, which unwind the 5' mRNA cap of the mRNA: this structure contains m⁷GpppN (7-methylguanosine-triphospho-5'-ribonucleoside) (Jackson *et al.*, 2010). This process prepares the mRNA for the attachment of ribosome. The complex of eIF4F consists of the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A and the scaffold protein eIF4G, which binds eIF4E, eIF4A, poly(A)-binding protein (PABP), eIF3 and Mnk1, leading to the association of the eIF4F and the mRNA. The 43S PIC is then attached to the eIF4F-mRNA complex (Pestova *et al.*, 2001; Silvera *et al.*, 2010), becoming 48S complex.

The 48S complex scans along the mRNA, and secondary structures in the untranslated region (UTR) are unwound by eIF4A helicases (Sonenberg and Hinnebusch, 2009). When a start codon (usually AUG) is recognized, it base-pairs with the anticodon of the initiator tRNA, allowing 60S ribosomal subunit to join with 40S subunit. For them to form an 80S ribosome, eIF5B effectively dissociates eIFs from the complex (Hinnebusch, 2006; Jackson *et al.*, 2010). Then the joining of a 60S ribosomal subunit and the 40S ribosomal subunit produces an active 80S ribosome for the elongation phase (Meric and Hunt, 2002; Jackson *et al.*, 2010).

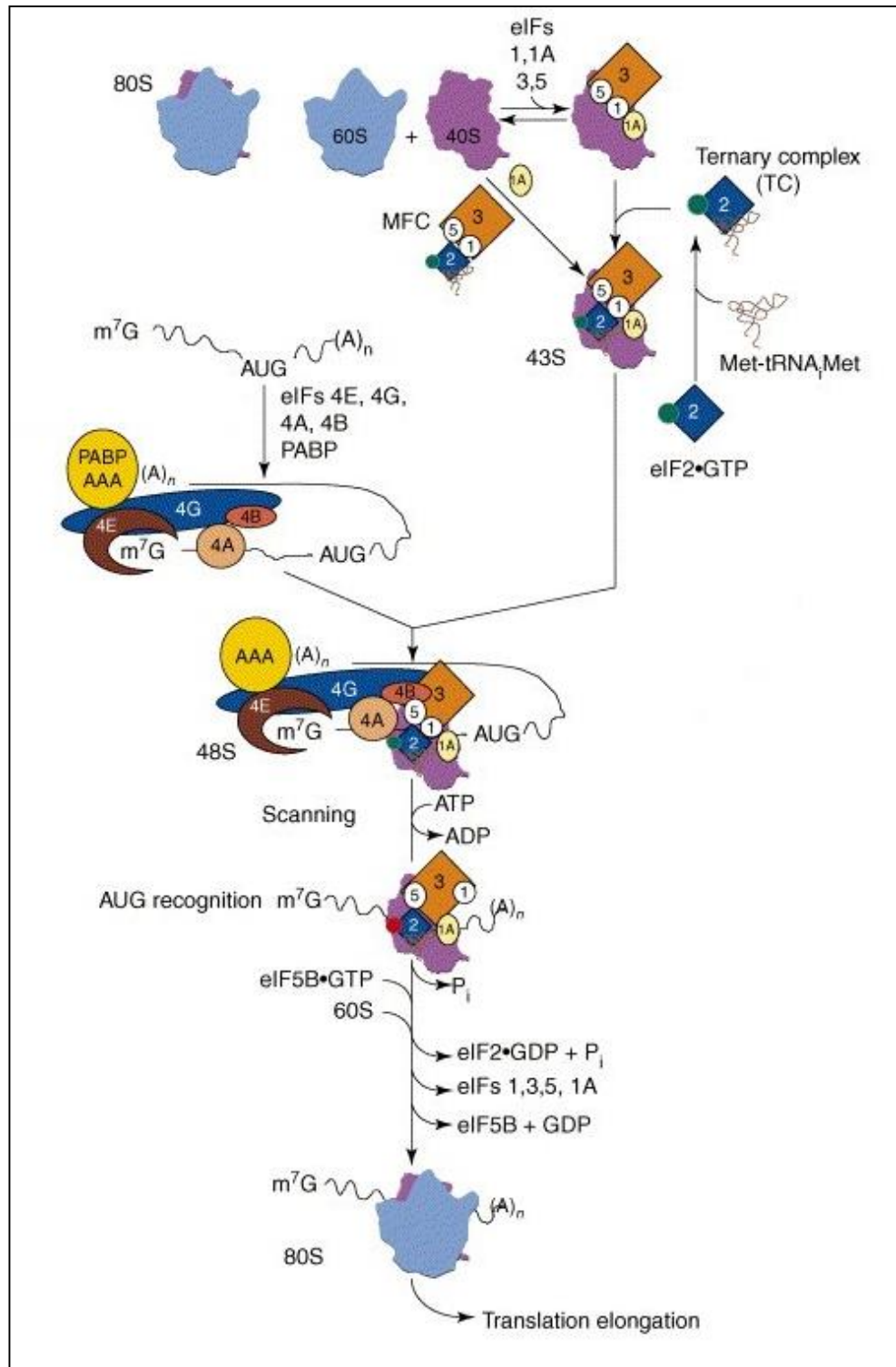


Figure 1.1 Mechanism of eukaryotic initiation translation. This shows the formation of 43S preinitiation complex (PIC) which requires ternary complex (eIF2-GTP-Met-tRNA_i) and multifactor complex. Then 48S complex scans the mRNA in order to recognise a start codon. The 40S subunit then joins with the 60S ribosomal subunit in a reaction requiring a number of eIFs and GTP. Adapted from Hinnebusch (2006).

1.3 The composition and function of the eIF4F complex

There are several eukaryotic initiation translation factors (eIFs) which are required in the initiation steps (shown in Table 1.1). This section focuses on the details of components of eIF4F complex, namely the 46 kDa helicase eIF4A, the 24 kDa phosphoprotein eIF4E and the 220 kDa scaffold protein eIF4G. Formation of the eIF4F complex is essential for cap-dependent translation (Gingras *et al.*, 1999; Holcik and Sonenberg, 2005; Sonenberg and Hinnebusch, 2009). It is also believed to be the rate limiting step of translation initiation (Gingras *et al.*, 1999).

Table 1.1 A list of main eukaryotic initiation translation factors.

Factors	Size (kDa)	Function	Reference
eIF1	12	Stimulates binding of eIF2-GTP-Met-tRNA _i to the 40S ribosomal subunit and promotes the recognition of the start codon	(Passmore <i>et al.</i> , 2007; Jackson <i>et al.</i> , 2010)
eIF1A	17	Stimulates binding of eIF2-GTP-Met-tRNA _i to the 40S ribosomal subunit and aids eIF1 in the recognition of the start codon	(Passmore <i>et al.</i> , 2007; Jackson <i>et al.</i> , 2010)
eIF2	125.6 (3 subunits)	When associated with GTP, eIF2 binds Met-tRNA _i to form eIF2 ternary complex	(Kimball, 1999; Kimball, 2002)
eIF2B	294 (5 subunits)	A guanosine nucleotide exchange factor that exchanges eIF2-associated GDP with GTP	(Gomez <i>et al.</i> , 2002; Jennings <i>et al.</i> , 2013)
eIF3	800 (13 subunits)	Stabilises the above complex and also the cap-binding complex	(LeFebvre <i>et al.</i> , 2006; Wagner <i>et al.</i> , 2014)

Factors	Size (kDa)	Function	Reference
eIF4A	46	Unwinds the structure of the 5'UTR	(Rogers <i>et al.</i> , 1999; Rogers <i>et al.</i> , 2002)
eIF4B	70	Stimulates the helicase activity of eIF4A	(Raught <i>et al.</i> , 2004; Rhoads, 2009)
eIF4E	24.5	Binds the 5' cap of mRNA	(Rhoads, 2009)
eIF4G	220	Binds eIF4A, eIF4E, eIF3, PABP and mRNA	(Lamphear <i>et al.</i> , 1995; Willcocks <i>et al.</i> , 2004; Yanagiya <i>et al.</i> , 2009)
eIF4H	25.4	Stimulates the helicase activity of eIF4A	(Rogers <i>et al.</i> , 2001)
eIF5	58	Facilitates the binding of eIF2-GTP-Met-tRNA _i to the 40S ribosomal subunit and promotes in the AUG recognition	(Passmore <i>et al.</i> , 2007)
eIF5B	139	Promotes ribosomal subunit joining and displacement of eIF2-GDP and factors	(Pestova <i>et al.</i> , 2001; Jackson <i>et al.</i> , 2010; Pisareva and Pisarev, 2014)

1.3.1 eIF4A

Eukaryotic translation initiation factor 4A (eIF4A) is a DEAD-box RNA helicase (Rogers *et al.*, 2002) that is responsible for unwinding the secondary structure in the 5'UTR of the mRNA with a requirement for ATP. This allows ribosome subunits to bind the mRNA and subsequently scan AUG start codons on the mRNA. (Rogers *et al.*, 1999; Oberer *et al.*, 2005; Parsyan *et al.*, 2011). The activity of eIF4A is enhanced by the co-factors eIF4B and eIF4H (Gingras *et al.*, 1999; Rogers *et al.*, 2001; Raught *et al.*, 2004). eIF4A is named a DEAD box helicase because it contains conserved amino acids, namely conserved aspartic acid, glutamic acid, alanine and aspartic acid (DEAD) (Rogers *et al.*, 2002). The amino acid sequence of eIF4A (Figure 1.2) consists of nine motifs, which are conserved in the DEAD-box helicase family—namely motif Q, motif I, motif Ia, motif Ib, motif II, motif III, motif IV, motif V, and motif VI (Schutz *et al.*, 2008; Lu *et al.*, 2014). Motifs Q, I, and II are responsible for ATP hydrolysis, while motifs Ia, Ib, III, IV, V and VI are involved in the interaction with, or the binding to RNA (Tanner *et al.*, 2003; Cordin *et al.*, 2006).

In humans, there are three eIF4A isoforms characterised namely eIF4AI, eIF4AII and eIF4AIII (Parsyan *et al.*, 2011). At the amino acid level, eIF4AI and eIF4AII share 90-95% sequence identity. These two forms are involved in translational activity (Andreou and Klostermeier, 2013). They are defined as being RNA-dependent ATPases and as having ATP-dependent activity (Parsyan *et al.*, 2011). For the different functions of eIF4AI and eIF4AII, it has been shown that suppression of eIF4AI increases transcription of eIF4AII, leading to higher eIF4AII mRNA and protein levels. However, there is no significant change in eIF4AI mRNA levels after eIF4AII is knocked down. This suggests that there may be differences in the cellular functions of eIF4AI and eIF4AII (Galicia-Vazquez *et al.*, 2012; Andreou and Klostermeier, 2013). eIF4AIII shows ~60% identity with these two isoforms (Li *et al.*, 1999; Lu *et al.*, 2014) and its function is not related to translation (Lu *et al.*, 2014). In contrast to the translational function of eIF4AI and eIF4AII, eIF4AIII is involved in exon junction complex formation following mRNA splicing (Parsyan *et al.*, 2011; Lu *et al.*, 2014).



Figure 1.2 Alignment of human eIF4A1, eIF4A2 and eIF4A3. This shows the conserved residues (red) and divergent residues (blue). This also shows where each motif of eIF4A is. This figure was taken from Lu *et al.* (2014).

1.3.2 eIF4E

eIF4E is a 24 kDa phosphoprotein that was first identified as binding to the 5' cap of most mRNAs with high affinity during translation initiation (Sonenberg and Gingras, 1998; Tomoo *et al.*, 2003; Rhoads, 2009). It has roles in controlling cell growth and proliferation (Sonenberg and Gingras, 1998). The level of expression of eIF4E is low and the amount of expressed eIF4E is the lowest compared to the amount of other eIFs in most cell types (Gingras *et al.*, 1999). Crystallographic and NMR studies have been used to analyse the structure of eIF4E and the mRNA cap from yeast (Matsuo *et al.*, 1997) and murine proteins (Marcotrigiano *et al.*, 1997). In 2003, the crystal structures of human eIF4E bound to the cap analogues, m⁷GTP and m⁷GpppA, were elucidated (Tomoo *et al.*, 2003) and revealed that the shape of eIF4E is like a cupped hand or baseball glove (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). The 5' cap of the mRNA is bound in the concave surface of eIF4E (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997; Tomoo *et al.*, 2003).

The cap-binding ability of eIF4E is negatively regulated by interaction with its protein inhibitors, the eIF4E binding proteins (4E-BPs) (Gingras *et al.*, 2001; Hinnebusch, 2012). There are three isoforms of 4E-BPs in mammals, comprising 4E-BP1, 4E-BP2, and 4E-BP3 (Yanagiya *et al.*, 2012). eIF4E associates with a specific binding motif on the 4E-BPs (YXXXXLΦ, where X is any amino acid and Φ is Leu, Met, or Phe). This motif is also present on eIF4G, which is able to interact with eIF4E (Marcotrigiano *et al.*, 1997; Rhoads, 2009). Thus the 4E-BPs are involved in translational regulation by association with eIF4E which then leads to the inhibition of eIF4E bound to eIF4G in order to form the eIF4F complex (Hinnebusch, 2012). The association of eIF4E and the 4E-BPs is controlled by mammalian target of rapamycin (mTOR)-dependent phosphorylation which is activated by extracellular stimuli e.g. hormones, growth factors, or mitogens (Gingras *et al.*, 2001). Briefly, hypophosphorylated 4E-BP1 can bind strongly to eIF4E while eIF4E-binding 4E-BP1 is phosphorylated at Thr37 and Thr46 residues, priming the phosphorylation of Thr70 and Ser65 in order to release free eIF4E to interact with other factors in the eIF4F complex (Gingras *et al.*, 2001; Sonenberg and Hinnebusch, 2009).

1.3.3 eIF4G

eIF4G is a 220 kDa scaffold protein that possesses docking sites for interaction with its partners. There are three characterised forms of mammalian eIF4G; eIF4GI, eIF4GII and eIF4GIII (DAP5, death-association protein 5). The first two isoforms share 46% homology (Coldwell *et al.*, 2012). eIF4GI is the most abundant form of eIF4G (approximately 85% of eIF4G). Each eIF4G isoform has a different biochemical activity (Coldwell *et al.*, 2004; Coldwell and Morley, 2006; Hinton *et al.*, 2007). eIF4GI consists of 1,600 amino acids (Bradley *et al.*, 2002; Fraser, 2009) and contains binding sites for several binding partners (Figure 1.3). The N-terminus of eIF4G interacts directly with poly-A binding protein (PABP) and eIF4E (Coldwell *et al.*, 2012). PABP is a translation initiation protein, involved in mRNA circularisation (Sonenberg and Hinnebusch, 2009). PABP associates with the 3' poly(A) tails (Kahvejian *et al.*, 2005), and this increases the affinity of eIF4E for m⁷GTP cap structures (Borman *et al.*, 2000; Kahvejian *et al.*, 2005).

The conserved middle region of eIF4G contains a binding site for eIF4A (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997; Marintchev and Wagner, 2004) and another site for interaction with eIF3 (Lamphear *et al.*, 1995; Morino *et al.*, 2000; LeFebvre *et al.*, 2006). This central region of eIF4G also possesses RNA-binding activity (Yanagiya *et al.*, 2009). Its crystal structure demonstrates that it folds into 5 HEAT motifs (Marcotrigiano *et al.*, 2001). The C-terminal region of eIF4G contains a second, independent binding site for eIF4A (Imataka and Sonenberg, 1997; Korneeva *et al.*, 2001) and a MAPK signal integrating kinase (Mnk1)-binding domain (Lamphear *et al.*, 1995; Pyronnet *et al.*, 1999). Mnk1 is one of a member of serine/threonine kinases which can bind to the C-terminus of eIF4G (Joshi and Platanias, 2014). Once the association of eIF4G and Mnk1 occurs, Mnk1 phosphorylates eIF4E on S209, leading to a decrease of its binding to the 5' cap of mRNA (Scheper *et al.*, 2002).

PTMs such as phosphorylation have been shown to form an important part in fine-tuning translation. For example, phosphorylation of eIF4GI at Ser1186 by protein kinase C α (PKC α) can modulate the binding of eIF4G to Mnk1 which is an eIF4E kinase. Mutation of the specific eIF4GI phosphorylation site affects binding to Mnk1. This mutation leads to the failure of translational control through the phosphorylation cascade of eIF4E and other eIF4G partners (Dobrikov *et al.*, 2011).

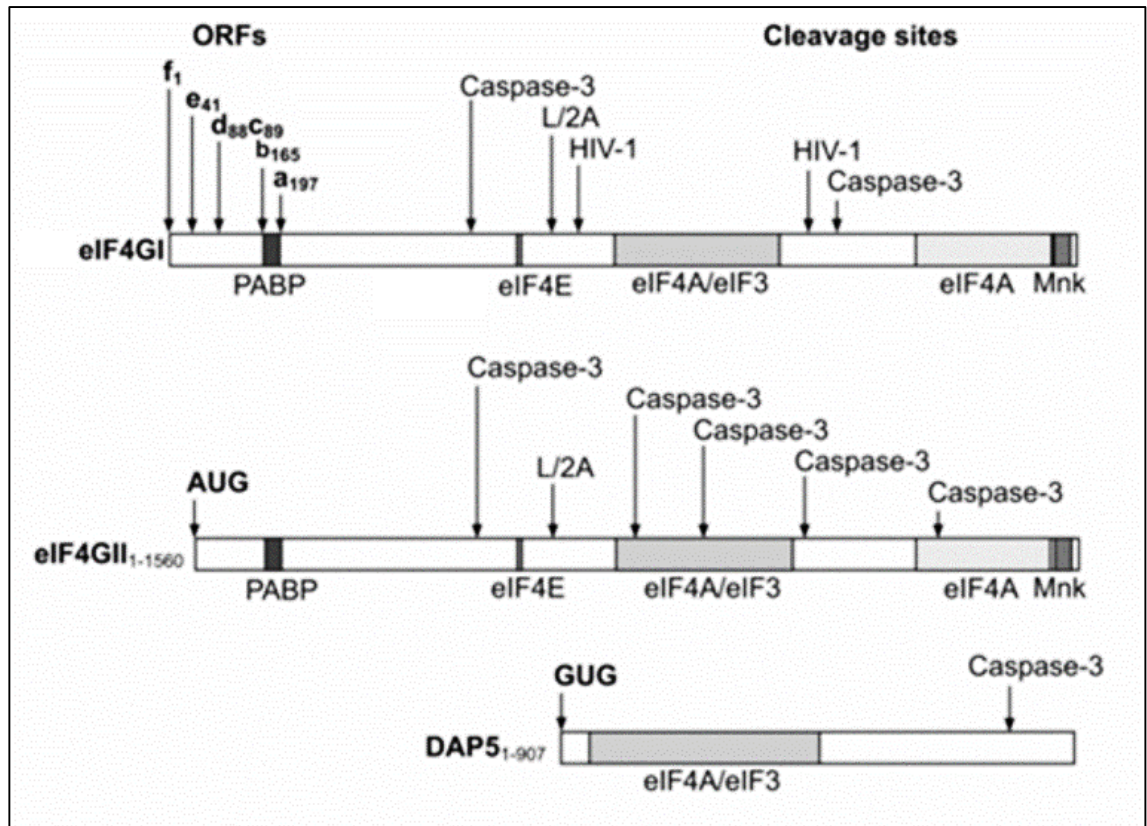


Figure 1.3 Schematic representation of eIF4GI, eIF4GII and DAP5. This shows regions of binding sites of other components of the translation initiation machinery. This also shows different forms of eIF4GI which come from different start codons (AUG) (f-a). Several cleavage sites of caspase-3, viral proteases (L/2A) and HIV-1 proteases are indicated which provide different fragments of eIF4G during apoptosis or some viral transfection. Modified from Coldwell *et al.* (2012).

1.4 Regulation of translation

There are several mechanisms involved in controlling translation initiation. Some translational regulation involves the availability of eIFs, while in other cases regulation is at the level of the mRNA itself. Some examples of translational regulation are described below.

1.4.1 eIF2 and eIF2B regulation

As described in section 1.2, a complex of eIF2 and GTP specifically interacts with Met-tRNA_i to bring about interaction with the 40S ribosomal subunit by the formation of the eIF2 ternary complex, and is released as an inactive complex (eIF2-GDP). As shown in Figure 1.4, the α -subunit of eIF2 is phosphorylated on Ser51 by

serine/threonine kinases during stress (Kimball, 2002). Phosphorylated eIF2 α is a competitive inhibitor of eIF2B (act as a guanine-nucleotide exchange factor, GEF), which is required to exchange GDP for GTP (Sonenberg and Hinnebusch, 2009; Le Quesne *et al.*, 2010). Thus, Met-tRNA_i binding to eIF2-GTP is decreased, resulting in an inhibition of 43S PIC formation that subsequently arrests protein synthesis (Kimball, 2002; Le Quesne *et al.*, 2010). This is consistent with overexpression of a non-phosphorylatable form of eIF2 α (Ser51Ala), which can cause malignant transformation (Donze *et al.*, 1995). This suggests that regulation of eIF2 and eIF2B is an important mechanism for global protein synthesis and cell homeostasis (Klann and Dever, 2004; Hershey, 2010).

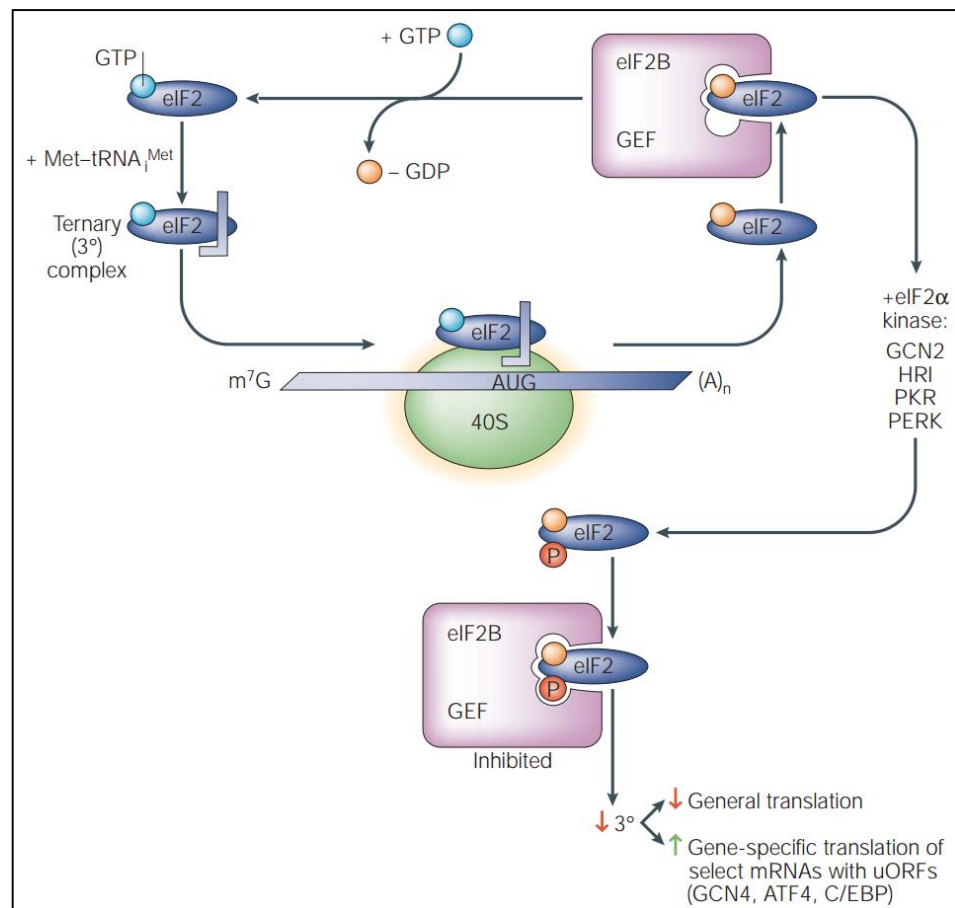


Figure 1.4 Translational regulation via eIF2 and eIF2B. The activity of the eIF2 ternary complex (eIF2-GTP-Met-tRNA_i) is controlled by the guanine nucleotide exchange factor eIF2B, which exchanges eIF2-associated GDP with GTP. There are four mammalian kinases, namely GCN2, HRI, PKR, and PERK that phosphorylate the α -subunit of eIF2 at serine 51. (Klann and Dever, 2004; Hershey, 2010). This figure is taken from Klann and Dever (2004).

1.4.2 eIF4E regulation

In eukaryotes, controlling the rate of translation initiation is dependent on the cap binding activity of eIF4E. There are several mechanisms involved (reviewed in Sonenberg and Hinnebusch, 2009). For example, one of the mechanisms is a cap-binding ability of eIF4E which is inhibited by the members of the 4E-BP family (Figure 1.5). This mechanism involves the competition of 4E-BPs with eIF4G for association with eIF4E and results in translational repression as described in section 1.3.2. Specifically, 4E-BP1 which is hypophosphorylated can bind strongly to eIF4E, while 4E-BP1 which is highly phosphorylated via the PI3K/AKT/mTOR pathway releases free eIF4E, allowing it to interact with other factors in the eIF4F complex (Sonenberg and Hinnebusch, 2009). In another regulatory process, eIF4E is phosphorylated on Ser209 by Mnk1 that binds to the C-terminus of eIF4G. Mnk1 is activated through the MAP kinase/ERK pathway. This phosphorylation cascade can occur even if eIF4E binds to 4EBP1 (Waskiewicz *et al.*, 1999). Another example is the competition of eIF4E homolog (4E-HP) that binds directly to the 5' cap structure of mRNA. This competitively prevents eIF4E from binding to the cap structure, resulting in translational repression of specific mRNAs (Sonenberg and Hinnebusch, 2009).

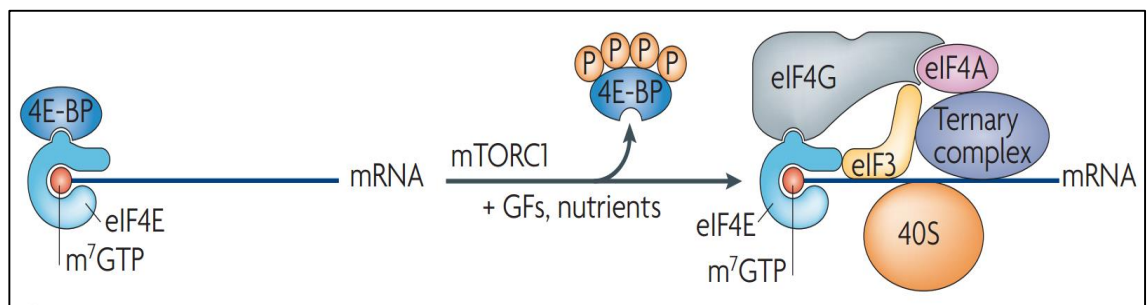


Figure 1.5 Translational regulation via eIF4E and 4E-BPs. Hyperphosphorylation of 4E-BPs is activated via the PI3K/AKT/mTOR pathway, leading to release free eIF4E. This allows the association of eIF4E with other factors in the eIF4F complex. Modified from Ma and Blenis (2009).

1.4.3 Translational control by miRNAs (microRNAs)

MicroRNAs (miRNAs) are short non-coding RNAs containing ~21 oligonucleotides (Hershey, 2010; Jackson *et al.*, 2010). miRNAs bring about another mode of translational repression. In the mammalian genome, there are 800–1,000 genes transcribed into miRNAs, suggesting that they are important regulators of gene expression (Sonenberg and Hinnebusch, 2009; Hershey, 2010). miRNAs and associated proteins (called an RNA-induced silencing complex (RISC)) interact with a complementary site in a mRNA which is usually in the 3' UTR. This complex functions to inhibit translation from specific mRNAs (Hutvagner and Zamore, 2002). However, recent studies have provided models to describe how miRNA regulates translation. For example, a miRNA-mediated translational repression is proposed to occur through the recruitment of eIF4AII (Figure 1.6) (Izaurrealde, 2013; Meijer *et al.*, 2013). This involves the interaction of eIF4AII with NOT1 in the CCR4-NOT deadenylase complex and miRNA-induced silencing complex (miRISC), consisting of a miRNA associated with an Argonaute protein (AGO) and a GW182 protein (Izaurrealde, 2013). It is proposed that eIF4AII recruited by NOT1 binds to the 5'UTR, and then possibly blocks the start codon scanning of 43S PIC (Izaurrealde, 2013).

Another model (Figure 1.7) suggests that miRNA-mediated translational repression is involved in the association of the GW182 protein with cytoplasmic poly(A)-binding protein 1 (PABPC1). An Argonaute protein (AGO) in the miRISC associates with N-terminal GW-repeated domain of a GW182 protein. The middle (M) and C-terminal silencing domains of GW182 proteins bind to PABPC1, leading to translational repression and mRNA degradation. This is because the association of GW182 with PABPC1 competitively prevents eIF4G binding to PABPC1, affecting the reduction of translation. In addition, miRNA-silenced mRNA is deadenylated by the CAF1–CCR4–NOT1 deadenylase complex, and the 5'-cap is then removed by decapping protein 2 (DCP2). To have a fully enzymatic activity, DCP2 requires a decapping protein 1 (DCP1), an enhancer of decapping 4 (EDC4) and a putative RNA helicase DEAD-box protein 6 (DDX6) (Tritschler *et al.*, 2010).

Figure 1.6 Translational repression through miRNA-mediated pathway by recruiting eIF4AII. Interaction of a subunit NOT1 of CCR4-NOT deadenylase complex and miRISC, which consists of Argonaute proteins (AGO) associated with miRNAs and GW182 proteins, has a role in the translational repression through the recruitment of the DEAD-box RNA helicase eIF4AII (Izaurrealde, 2013; Meijer *et al.*, 2013), suggesting that eIF4AII may lock the 5'UTR of the mRNA and possibly prevent the ribosomal scanning (Izaurrealde, 2013).

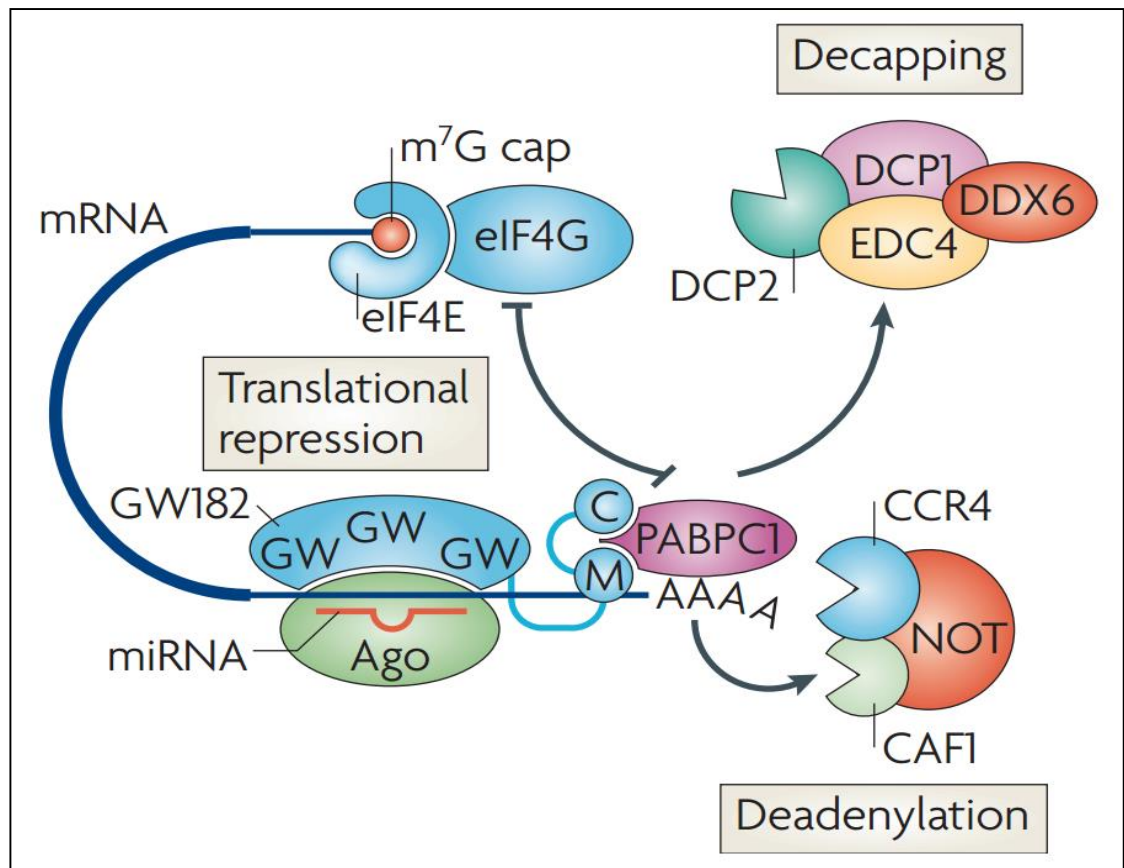


Figure 1.7 Translational repression through miRNA-mediated pathway by interacting of GW182 protein in complex and PABPC1. An Argonaute protein (Ago) in the miRISC associates with N-terminus of GW182 protein. The GW182 silencing domains, middle (M) and C-terminus (C), bind to PABPC1, leading to translational repression and mRNA degradation. It is as a result of the association of GW182 with PABPC1 that competitively prevents eIF4G binding to PABPC1. Therefore this leads to the translational repression and subsequently deadenylation. Deadenylation of miRNA-silenced mRNA requires the activity of the CAF1–CCR4–NOT1 deadenylase complex. The 5'-cap structure of the silenced mRNA is also removed by decapping protein 2 (DCP2) with the associated proteins, namely a decapping protein 1 (DCP1), an enhancer of decapping 4 (EDC4) and a putative RNA helicase DEAD-box protein 6 (DDX6) (Tritschler *et al.*, 2010).

1.4.4 Translational control by processing bodies and stress granules

Cytoplasmic mRNA that is not translated by ribosomes can be present in either processing bodies (P-bodies) or stress granules (SGs) through the action of specific RNA-binding proteins. The mRNA within both structures is in dynamic equilibrium with mRNA in polysomes. In terms of translational regulation, it is observed that stress-induced translational repression results in decreased mRNA within polysomes, while the mRNA increases in P-bodies and SGs (Kedersha and Anderson, 2009; Decker and Parker, 2012).

1.4.5 Translational control by alternative pathways

Translational regulation by the formation of protein-RNA complexes can also occur via the 5'UTR of the mRNA. A well-characterized example is ferritin mRNA. In the absence of iron, translation of ferritin mRNA is inhibited by the iron regulatory element (IRE)-binding protein (IRP). The association of IRP with ferritin mRNA occurs at the 5' cap structure which strongly inhibits the 43S complex from being loaded onto the mRNA (Gebauer and Hentze, 2004; Hershey, 2010). Other examples include regulation by specific 3' UTR-protein interactions and regulation by trans-acting proteins [reviewed in Sonenberg and Hinnebusch (2009); Hershey (2010); Jackson *et al.* (2010)].

1.5 Translation and cancer

Tumorigenesis can occur through the disruption of a number of different cellular processes, which includes DNA damage repair and protein synthesis (Watkins and Norbury, 2002). Hypothetically, a translational apparatus that is moderately repressed can create a balance of protein synthesis, allowing cells to regulate their proliferation. In contrast, over-activation of translational complexes leads to an imbalance of protein production, resulting from "weak" mRNAs being translated relatively more efficiently. The "weak" mRNA-encoded proteins are involved in facilitating cell growth and proliferation. The dysregulated increase in the levels of those proteins causes cells to become malignant (Hershey, 2010). Specifically, the alteration in the levels of eIFs results in abnormal translation of specific mRNAs and consequently abnormal cell growth, possibly leading to cancer (Yin *et al.*, 2011). Many studies on the control of translation have shown a correlation between altered expression of eIFs and tumour formation Table 1.2.

Table 1.2 Eukaryotic translation initiation factors related to human cancers

Factor	Status	Tumour type	Reference
eIF2a	Protein increase	Non-Hodgkin's lymphoma Gastro-intestinal	(Wang <i>et al.</i> , 1999) (Lobo <i>et al.</i> , 2000)
eIF3a	Protein increase	Lung, Breast, Cervical, Esophageal	(Pincheira <i>et al.</i> , 2001)
eIF3b	Protein increase	Breast	(Lin <i>et al.</i> , 2001)
eIF3c	mRNA increase	Testicular	(Rothe <i>et al.</i> , 2000)
eIF3h	mRNA increase	Prostate and Breast	(Nupponen <i>et al.</i> , 1999)
eIF4AI	mRNA increase	Melanoma Liver	(Eberle <i>et al.</i> , 1997) (Shuda <i>et al.</i> , 2000)
eIF4E	mRNA increase Protein increase	Liver Bladder Colon, Head & Neck, Breast Bladder, Lung, Prostate, Hodgkin's lymphoma	(Shuda <i>et al.</i> , 2000) (Berkel <i>et al.</i> , 2001) (Wang <i>et al.</i> , 1999) (Mamane <i>et al.</i> , 2006)
eIF4GI	Gene amplification	Squamous cell lung	(Gingras <i>et al.</i> , 1999; Bauer <i>et al.</i> , 2002)
eIF5AII	Gene amplification	Ovarian	(Guan <i>et al.</i> , 2001)

Note: This table is modified from previous publications (Watkins and Norbury, 2002; Rajasekhar and Holland, 2004)

1.6 Post-translational modification

Post-translational modifications (PTMs) of proteins are biological processes occurring after protein synthesis whereby translated polypeptides are modified to achieve full function. PTMs involve the addition of functional groups, proteins or peptides. They

include phosphorylation, methylation, acetylation, ubiquitylation and small ubiquitin-like modification (SUMOylation). PTMs have crucial roles in the regulation of cellular processes, altering the biological activities or conformational structures of the polypeptides. PTMs related to this study will be described below.

1.6.1 Phosphorylation

The first PTM identified and functionally characterized was phosphorylation (Burnett and Kennedy, 1954). Phosphorylation is a mainly reversible process that acts by adding a phosphate (PO_4) molecule to a polypeptide by one of many kinases. It occurs in both prokaryotes and eukaryotes. It plays important roles in the control of cellular processes such as cell cycle, cell growth, apoptosis and protein synthesis. Enzymes can be turned on and off by phosphorylation (kinases) and dephosphorylation (phosphatases). For example, phosphorylation can lead to a conformational change in protein structure, causing the protein to become activated or deactivated forms. In eukaryotic organisms, phosphorylation usually occurs on serine, threonine and tyrosine residues. For instance, the phosphorylation of eIF4E at Ser209 decreased the affinity of eIF4E for the m^7GTP cap structure of the mRNA (Scheper *et al.*, 2002) and is likely to be important in regulation of mRNA transport and the transformation activity of eIF4E (Topisirovic *et al.*, 2004).

1.6.2 Ubiquitylation

Ubiquitin is an 8.5 kDa regulatory protein that is ubiquitously present in almost all eukaryotic cells. It is highly conserved in eukaryotes. There are only three amino acid differences between human and *S. pombe* ubiquitin. Ubiquitin can be covalently attached through an isopeptide bond to lysine residues in target proteins. Ubiquitylation or ubiquitination has roles in proteolysis and modification of protein function. This ubiquitin modification can occur as either a single ubiquitin (monoubiquitylation) or by an ubiquitin chain (polyubiquitylation). The mechanism occurs via a cascade through the activity of a number of proteins, the E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) proteins (Figure 1.8). Ubiquitin is produced as a precursor protein which needs to be processed to the mature form by specific ubiquitin proteases in order to reveal a diglycine (GG) motif at the C-terminus. Ubiquitin-GG is then activated in an ATP-dependent reaction by the formation of a thioester bond with the E1 activating enzyme. Next, it is passed to an E2 ubiquitin

conjugating enzyme to form another thioester bond. In many cases ubiquitin can attach directly to target proteins, but in some cases ubiquitin requires one of a large number of E3 ubiquitin ligases prior to the attachment with targets since the E3s provides the specificity for the modification. Ubiquitin can be removed from targets by the actions of deubiquitinating enzymes (DUBs) (Watts *et al.*, 2014).

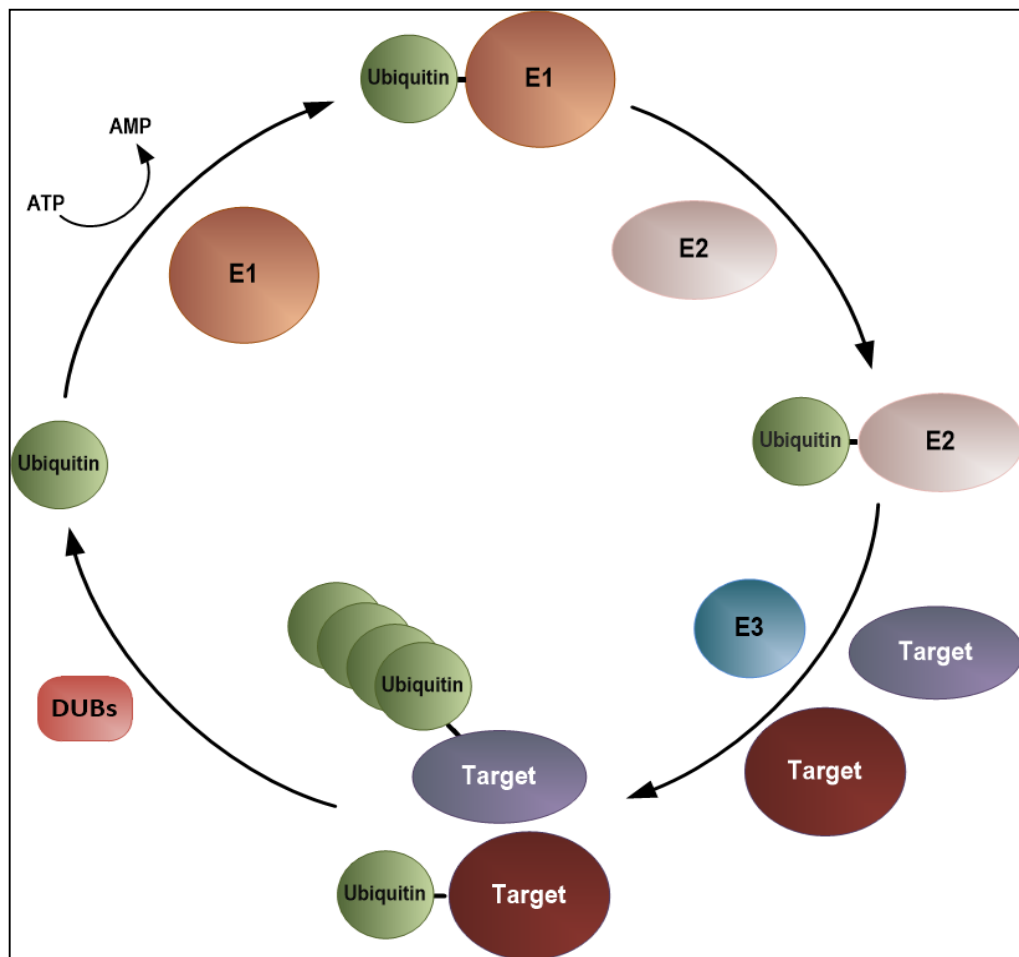


Figure 1.8 Ubiquitylation pathway. E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, E3 ubiquitin ligase, DUB deubiquitylating enzyme. Ubiquitin is activated by the formation of an ubiquitin-adenylate before forming a thioester bond with a cysteine residue in the E1 ubiquitin activating enzyme. Ubiquitin is passed to an E2 ubiquitin conjugating enzyme, again forming a thioester bond. Target proteins are recognized by E3 ubiquitin ligases, either directly or via an adaptor, and ubiquitin is attached via the formation of an ϵ -amino bond. Ubiquitin can be attached to target proteins either as a monomer, or in the form of ubiquitin chains. Ubiquitin can be removed from target proteins by the action of one of a number of DUBs (Watts *et al.*, 2014).

1.6.3 SUMOylation

SUMO is a small ubiquitin-like molecule that is attached post-translationally to target proteins. SUMOylation is an essential process in most organisms including eukaryotes such as yeast, plants and animals (Johnson, 2004; Talamillo *et al.*, 2008). SUMOylation has roles in the response to DNA damage, cell signalling, cell cycle and RNA processing; in particular, SUMOylation can affect protein-protein interactions, protein localisation, enzyme activity and protein stability. In humans, there are four SUMO proteins; SUMO1, SUMO2, SUMO3 and SUMO4. The expression of SUMO1, 2 and 3 appears to occur in all tissues while SUMO4 seems to be restrictedly expressed in spleen and kidney (Meulmeester and Melchior, 2008). However whether SUMO4 is conjugated to target proteins is not clear (Dohmen, 2004; Hay, 2005; Meulmeester and Melchior, 2008).

Like ubiquitin, SUMO is produced as a precursor protein that is cleaved to the mature form to reveal a diglycine motif at the C-terminus, by a SUMO-specific protease (mammalian: SENP1; yeast: Ulp1) (

Figure 1.9 and Table 1.3). It is then activated by the formation of a thioester bond with a heterodimeric SUMO E1 activating enzyme (e.g. human: Aos1-Uba2; *S. pombe*: Rad31-Fub2, see Table 1.3) in an ATP-dependent reaction. SUMO is then passed to the SUMO E2 conjugating enzyme (human: Ubc9; *S. pombe*: Hus5, see Table 1.3), forming another high energy thioester bond. From here SUMO can be directly attached to target proteins by the formation of an isopeptide bond between the C-terminal glycine residue and one or more lysine residues on the target. In some, but not all cases, this SUMOylation requires the presence of a specific SUMO E3 ligase (human: RanBP2 and PIAS family; *S. pombe*: Pli1, Nse2, see Table 1.3). Like ubiquitin, SUMO is found on targets either as monomeric SUMO protein or multimeric chains (Vertegaal *et al.*, 2006; Ulrich, 2008). SUMO2/3, but not SUMO1, form SUMO chains (Meulmeester and Melchior, 2008). SUMO can be removed from a modified target protein by the action of further SUMO-specific proteases such as Ulp1, Ulp2 in yeast or SENP1, 2, 3, 5, 6 and 7 in mammals (Table 1.3).

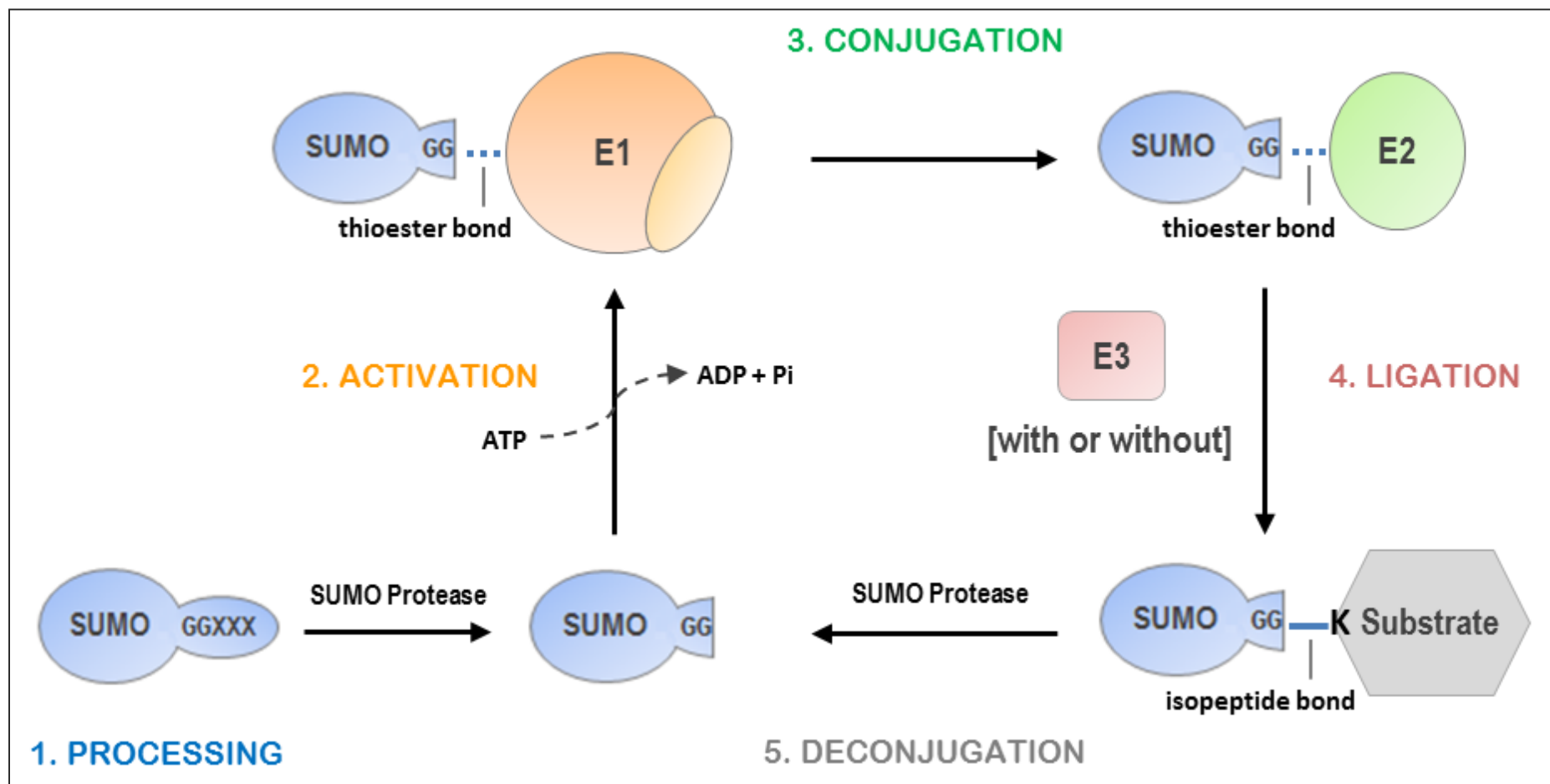


Figure 1.9 Cycle of SUMOylation. It shows five main steps namely processing, activation, conjugation, ligation and deconjugation. SUMO-GGxxx represents SUMO precursor, SUMO-GG represents matured SUMO, E1 represents SUMO activating enzyme, E2 represents SUMO conjugating enzyme and E3 represents SUMO ligases. This figure is modified from Hay (2005).

Table 1.3 List of components of the SUMOylation pathway in different species. (Adapted from Dr. Brenda Mercer, University of Sussex).

Component	<i>S. pombe</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>
SUMO	Pmt3 (Tanaka <i>et al.</i> , 1999)	Smt3 (Meluh and Koshland, 1995)	SUMO1 (Matunis <i>et al.</i> , 1996) SUMO2/3 (Lapenta <i>et al.</i> , 1997) SUMO4 (Bohren <i>et al.</i> , 2004)
Activating enzyme	Fub2/Rad31 (Shayeghi <i>et al.</i> , 1997)	Aos1/Uba2 (Johnson <i>et al.</i> , 1997)	SAE1/SAE2 (Gong <i>et al.</i> , 1999)
SUMO conjugating enzyme	Hus5 (al-Khodairy <i>et al.</i> , 1995)	Ubc9 (Johnson and Blobel, 1997)	Ubc9 (Desterro <i>et al.</i> , 1997)
Ligases	Pli1 (Xhemalce <i>et al.</i> , 2004) Nse2 (Andrews <i>et al.</i> , 2005)	Siz1/2 (Johnson and Gupta, 2001) Mms21/Nse2 (Zhao and Blobel, 2005) Zip3 (Cheng <i>et al.</i> , 2006a)	PIAS1 (Liu <i>et al.</i> , 1998) PIAS3 (Chung <i>et al.</i> , 1997) PIASy (Gross <i>et al.</i> , 2001) Mms21/Nse2 (Potts and Yu, 2005) Pc2 (Kagey <i>et al.</i> , 2003) RanBP2 (Pichler <i>et al.</i> , 2002) TOPORS (Weger <i>et al.</i> , 2005)
Protease Isopeptidases	Ulp1 (Taylor <i>et al.</i> , 2002) Ulp2 (Jongjitwimol <i>et al.</i> , 2014)	Ulp1 (Takahashi <i>et al.</i> , 2000) Ulp2 (Li and Hochstrasser, 2000)	SEN1 (Gong <i>et al.</i> , 2000) SEN2-8 (Gong and Yeh, 2006)

1.7 Substrates in SUMOylation

SUMO is often attached to substrates at a lysine residue in a consensus Ψ KXE motif (where Ψ is a large hydrophobic residue and X is any amino acid), but to date it is clear that lysine residues present in other sequence contexts can be modified by SUMO as well. Many SUMO targets have been identified and characterized in several cellular processes. In this section, some examples of SUMO targets will be given; specifically RanGAP, PCNA, TDG and PML.

1.7.1 RanGAP

RanGAP was the first SUMO target to be identified. It is modified by SUMO1 (Saitoh *et al.*, 1998). RanGAP1 is a trafficking protein that activates the GTPase Ran that is involved in nucleocytoplasmic transport of macromolecules and mitotic function. SUMOylation of RanGAP1 is required for its localisation at the nuclear pore complex and for association with Ubc9 and RanBP2/Nup358 (Johnson, 2004).

1.7.2 PCNA

Proliferating cell nuclear antigen (PCNA) is essential for DNA replication, and is also required for DNA repair at DNA lesions (Shivji *et al.*, 1992; Moldovan *et al.*, 2007). SUMOylation of *S. cerevisiae* PCNA occurs on K164 and K127. In this yeast, PCNA SUMOylation at K164 requires the SUMO ligase E3 Siz1 *in vivo* and *in vitro* (Hoege *et al.*, 2002). SUMOylation of *S. cerevisiae* PCNA recruits the Srs2 helicase to sites of replication in order to prevent the association of Rad51 and homologous recombination (Papouli *et al.*, 2005; Pfander *et al.*, 2005; Watts, 2006; Burkovics *et al.*, 2013). This is an example of SUMO mediating protein-protein interactions.

1.7.3 TDG

The human thymine-DNA glycosylase (TDG) has an important role in the base-excision DNA repair (BER) of G:U and G:T mismatches. Briefly, when DNA contains T or U residues that mismatch with G, they can be recognized and released by TDG, resulting in the creation of an abasic (AP) site. To allow the downstream AP endonuclease (APE) to bind to damaged DNA, SUMOylation of TDG on K330 is required. This leads to C-terminal conformational changes of TDG and decreased affinity of TDG for the AP site reduces. Thus the nucleotide is then restored by APE and TDG may be recycled

for the next rounds of the recognition of mispaired bases (Huang and D'Andrea, 2006; Smet-Nocca *et al.*, 2011).

1.7.4 PML

Promyelocytic leukaemia protein (PML) is a nuclear phosphoprotein identified as a result of a chromosomal translocation t(15;17), associated with acute promyelocytic leukaemia (APL). SUMO modification of PML is essential for formation of PML nuclear bodies and recruitment of associated proteins e.g. Sp100, p53, CBP and Daxx. When unSUMOylatable mutants of PML are introduced into PML-deleted cells, the aggregation of mutant PML occurs. This leads to the failure of PML to localize to PML bodies (Zhong *et al.*, 2000).

1.8 SUMOylation and cancer

In recent years, several studies have demonstrated that the disruption of SUMOylation is associated with a range of different tumours (Jacques *et al.*, 2005; Cheng *et al.*, 2006b; Sarge and Park-Sarge, 2009; Driscoll *et al.*, 2010; Zhu *et al.*, 2010). However, the relationship between SUMOylation levels and cancer is likely to be complex. For example, the SUMO-specific protease SENP1 is over-expressed in both prostate cancer (Cheng *et al.*, 2006b) and thyroid oncocytic tumour (Jacques *et al.*, 2005). SENP1 also induces the proliferation of prostate epithelial cells (Bawa-Khalfe *et al.*, 2010). The levels of SUMO modification increase significantly in multiple myeloma samples, correlating with high production of the E1 and E3 enzymes (Driscoll *et al.*, 2010), while the SUMO E2 is over-expressed in lung adenocarcinomas (McDoniels-Silvers *et al.*, 2002) and ovarian tumours (Mo *et al.*, 2005).

1.9 Preliminary data and hypothesis

The Watts lab have been using the fission yeast, *S. pombe*, as a model organism for the analysis of the role of SUMOylation. They have shown that SUMO and SUMOylation are required for the response to DNA damaging agents e.g. (Shayeghi *et al.*, 1997; Andrews *et al.*, 2005). To further analyse the role of SUMOylation, a complex containing the SUMO specific protease Ulp2 was purified (Figure 1.10) (Dr. Min Feng, University of Sussex). Interestingly, the protein co-purified with a number of translation initiation

factors and proteins required for RNA stability (Table 1.4). Supporting the notion that these proteins are affected by SUMOylation are the observations that some of them have been found in early screens for SUMOylated proteins in mammalian cells (Hannich *et al.*, 2005; Xu *et al.*, 2010). Interestingly, the levels of the mammalian homologues of several of the translation factors that were identified (eIF2 α , eIF3a, eIF3c, eIF3h, eIF4G, eEF1A2) are up-regulated in cancer cells (Stumpf and Ruggero, 2011). The preliminary data presented a link between these proteins and SUMOylation. Moreover, a significant number of translation initiation factors in budding yeast (*Saccharomyces cerevisiae*, Sc), plants (*Arabidopsis thaliana*), insect cells (*Drosophila melanogaster*) and/or mammalian cells (human: Hs, rat) have been identified in proteomic screens as being SUMOylated (Table 1.5). To further analyse the role of SUMOylation of some of the eIFs, this work has been focussing on an investigation into whether certain eIFs are SUMOylated and initiating studies into determining the role of SUMOylation of one of the proteins in the eIF4F complex.

1.10 Aims of this study

The aim of this project was to determine whether components of the eIF4F complex are SUMOylated and to initiate studies to investigate the role of this modification. The first results chapter (Chapter 3) was an investigation of SUMOylation of some of the RNA binding proteins in *S. pombe*, specifically eIF4G, eIF3h and the La protein homolog (Sla1), to determine whether they are modified by SUMO *in vivo*. These experiments were then extended to mammalian cells. Before this could be undertaken, the effects of stress conditions on protein synthesis and general SUMOylation in a range of cell lines were first analysed which will be demonstrated in Chapter 4. Next, in Chapter 5 and Chapter 6, *in vivo* and *in vitro* SUMOylation assays were used to determine whether mammalian eIF4G and eIF4A are modified by SUMO. Immunofluorescence analysis was also undertaken to determine whether eIF4G and eIF4A colocalise with SUMO in response to stresses. Additionally, studies were initiated to investigate the *in vivo* role of SUMO modification of eIF4AII.

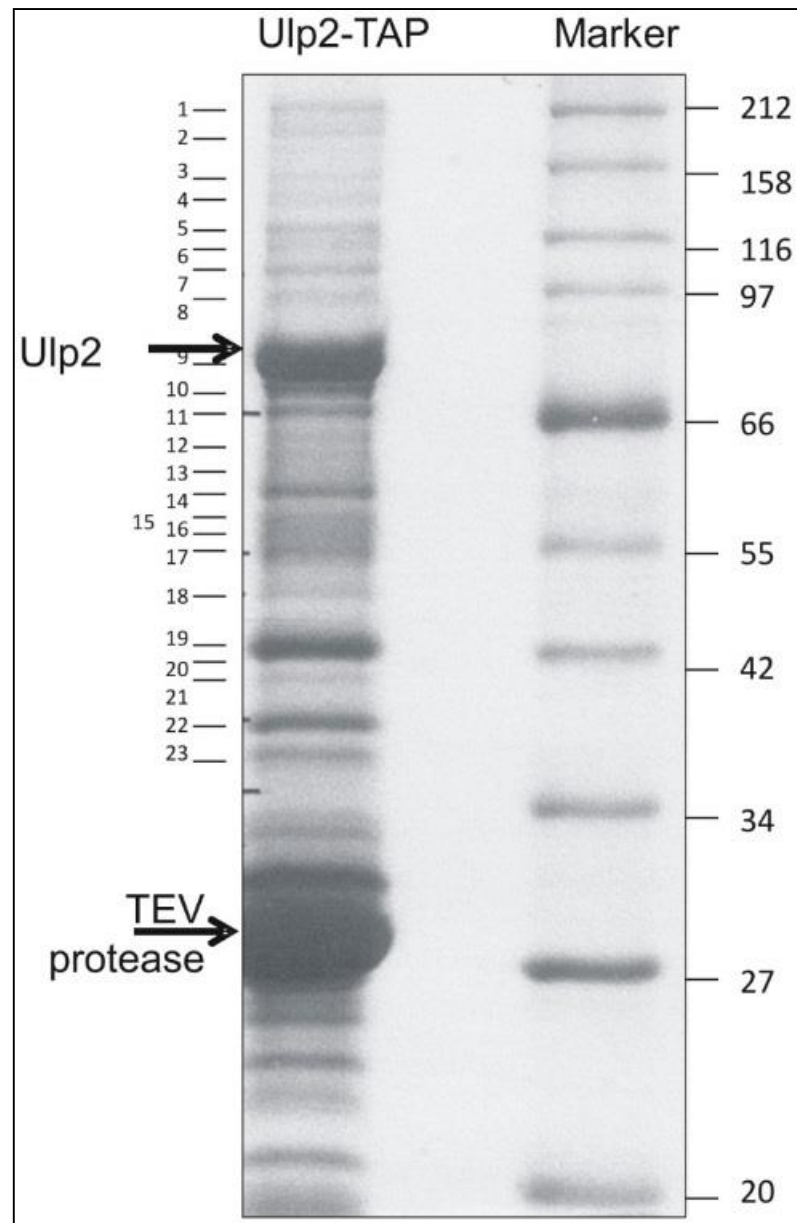


Figure 1.10 SDS-PAGE of Ulp2-Tap and associated proteins. TEV = TEV protease, used to cleave Ulp2 from TAP tag. Numbers refer to gel slices analysed by mass spectrometry (Dr. Min Feng, University of Sussex).

Table 1.4 Summary of proteins identified by mass spectrometry that co-purified with TAP-Ulp2.

Function	Protein
Translation	eIF2a, eIF2b, eIF3a, eIF3b, eIF3c, eIF3c, eIF3h, eIF4G, EF1a EF2B, eEF3B, EF2, Pabp
RNA synthesis	Rpa1, Rpa2
RNA processing	Rrp5, SPAC694.02, Exo2, Dhp1, Upf1, SPBC19G7.10C, Nop2, Dbp2, Prp19, Sla1
Ribosome biogenesis	aconitate hydrolase/mitochondrial ribosomal protein subunit L49, SPAC22G7.05, SPAC1142.04 (Noc2 predicted), Hsc1/Sks2, Rpl301, Rpl302, Rml2
DNA metabolism	Tcg1, Rfc5
Other	Pfk1, SPBC16h5.12C, glutamate 5-kinase (predicted), Gpd1, Gpd3

Table 1.5 Proteomic screens identified numerous SUMOylated eIFs.

Factor	SUMO	References
eIF1 α	Rat SUMO3	(Yang <i>et al.</i> , 2011)
eIF2A Subunit 1	Hs SUMO2	(Blomster <i>et al.</i> , 2009)
eIF2 Subunit α , γ	Drosophila SUMO	(Nie <i>et al.</i> , 2009)
eIF2B Subunit β	Hs SUMO1/2	(Westman <i>et al.</i> , 2010)
eIF2B	Plant SUMO	(Miller <i>et al.</i> , 2010)
eIF3A	Hs SUMO2	(Blomster <i>et al.</i> , 2009)
eIF3B	Hs SUMO2	(Blomster <i>et al.</i> , 2009)
eIF3C	Hs SUMO1/2	(Westman <i>et al.</i> , 2010)
eIF3D	Rat SUMO3	(Yang <i>et al.</i> , 2011)
eIF3E	Hs SUMO1/2	(Westman <i>et al.</i> , 2010)
eIF3I	Sc Smt3	(Panse <i>et al.</i> , 2004)
eIF3X	Hs SUMO2	(Blomster <i>et al.</i> , 2009)
eIF4AI	Drosophila SUMO Rat SUMO3 Hs SUMO2	(Nie <i>et al.</i> , 2009) (Yang <i>et al.</i> , 2011) (Blomster <i>et al.</i> , 2009; Bruderer <i>et al.</i> , 2011)
eIF4AII	Hs SUMO1	(Matafora <i>et al.</i> , 2009)
eIF4E	Hs SUMO1	(Xu <i>et al.</i> , 2010)
eIF4GI	Hs SUMO1/2	(Matafora <i>et al.</i> , 2009; Bruderer <i>et al.</i> , 2011)

Chapter 2

Materials and Methods

2.1 Yeast methods

2.1.1 Media

- 1) Yeast Extract (YE) – an enriched medium
 - 5 g/l yeast extract
 - 20 g/l glucose
 - 200 mg/l adenine
 - 100 mg/l leucine
 - 100 mg/l uracil
 - 100 mg/l histidine
 - 100 mg/l arginine
 - 25 g/l Difco (Bacto) agar (for making solid YE agar)
- 2) Yeast Nitrogen Base (YNB) – a selective medium
 - 1.9 g/l YNB (Formedium)
 - 4 g/l ammonium sulphate
 - 20 g/l glucose
 - 30 g/l Difco (Bacto) agar for making YNB agar (YNBA)
 - 0.08 g/l NaOH for making YNB agar (YNBA)
- 3) Extra Low Nitrogen (ELN) – a sporulation medium
 - 27.3 g/l Formedium Edinburgh Minimal Medium (EMM)
 - 50 mg/l ammonium chloride
 - 200 mg/l adenine
 - 100 mg/l leucine
 - 100 mg/l uracil
 - 100 mg/l histidine
 - 100 mg/l arginine
 - 25 g/l Difco (Bacto) agar

2.1.2 Media supplements

For some experiments the YNB broth or agar was supplemented. The media may contain some or all of supplements e.g. adenine, leucine and uracil, each at 100 mg/l. Thiamine was also used at 15 μ M for full repression (Cherkasova *et al.*, 2012).

2.1.3 *S. pombe* strains

S. pombe strains used in this study were shown in Table 2.1.

Table 2.1 List of *S. pombe* strains used in this study.

Strain	Genotype	References
<i>wt501</i>	<i>ade6-704, leu1-32, ura4-D18, h⁻</i>	(Murray <i>et al.</i> , 1991)
<i>sla1::ura(bs)</i>	<i>sla1::ura, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla1::ura(bs)</i>	<i>sla1::ura, ade6-704, leu1-32, ura4-D18, h⁺</i>	This study
<i>sla.wt</i>	<i>sla1.wt, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla.K106R</i>	<i>sla1.K106R, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla.K263R</i>	<i>sla1.K263R, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla.RR</i>	<i>sla1.K106R.K263R, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla1::kan(bs)</i>	<i>sla1::kan, ade6-M210, leu1-32, ura4-D18, h⁻</i>	This study
<i>sla1::kan(bs)*</i>	<i>sla1::kan, ade6-M210, leu1-32, ura4-D18, h⁺</i>	This study
<i>sla1::nat(bs)</i>	<i>sla1::Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>ulp2/eIF3b</i>	<i>ulp2-myc:kan, eIF3b-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>ulp2/eIF4G</i>	<i>ulp2-myc:kan, eIF4G-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>eIF3b-HA</i>	<i>eIF3b-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
<i>eIF4G-HA</i>	<i>eIF4G-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study

* Note: this strain was a kind gift from Dr. Stephanie Schalbetter, University of Sussex.

2.1.4 *S. pombe* plasmids

Two plasmids used in this study for the integration of mutations into the genome were pAW1 and the Cre recombinase expressing plasmid pAW8 (Watson *et al.*, 2008). The first plasmid pAW1 was used as PCR template because it contains the 'loxP–ura4⁺–loxM3' cassette. A pair of PCR primers was designed to amplify the cassette along with approximately 80-bp upstream and downstream sequences of target gene within the *S. pombe* genome.

The second vector pAW8 is derived from pUC19 vector and contains the *S. cerevisiae* LEU2⁺ gene which is controlled by the *nmt41* promoter. In the presence of thiamine, gene expression is not induced (no message in thiamine promoter). The plasmid also contains the *cre* gene (expresses bacteriophage P1 recombinase) which is also controlled by the *nmt1* promoter. Importantly, the plasmid has a multiple cloning site flanked by the loxP and loxM into which a coding sequence is cloned. The target gene cloned on this pAW8 can be mutated using site-directed mutagenesis.

2.1.5 Yeast transformation using lithium acetate

After yeast cells were grown overnight in 10 ml of YE medium, the culture was diluted into 40 ml of fresh YE for 3 hours and incubated at 30°C until mid-log phase. The culture was harvested by centrifugation at 3,000 rpm for 5 minutes. The pellet was washed with 10 ml of water and re-spun for 5 minutes. The pellet was then resuspended with 4 ml of 1x LiAc/TE buffer (0.1 M lithium acetate pH 7.5, 10 mM Tris pH 7.5, 1 mM EDTA). 1 ml of suspension cells was harvested by centrifugation at 13,000 rpm for 1 minute and resuspended in 100 µl of 1x LiAc/TE buffer. 20 µl (or 1 µg) of PCR product or plasmid was added and incubated at room temperature for 10 minutes. 260 µl of 40% polyethylene glycol (PEG) in 1x LiAc/TE buffer was added and the mixture was then incubated at 30°C for 1 hour. Subsequently, 43 µl of dimethyl sulfoxide (DMSO) was added and the cells were heat shocked at 37°C for 5 minutes. After the cells were harvested, the pellet was washed in 500 µl of sterile distilled water and resuspended once in 100 µl of remaining supernatant and plated onto YNBA plates supplemented with appropriate amino acids. Plates were incubated at 30°C for 3–5 days.

2.1.6 Recombination-mediated cassette exchange

The recombinase-mediated cassette exchange system (Watson *et al.*, 2008) was used for this study. To generate a base strain for a non-essential gene, the pAW1 vector was used as a PCR template. The PCR product containing the 'loxP-ura4⁺-loxM3' cassette flanked by upstream and downstream sequences of the gene was transformed into wild type *S. pombe* using lithium acetate-based yeast transformation following which the PCR product was integrated by homologous recombination. To create wild type and mutant alleles, a Cre-expression plasmid (pAW8) containing either the non-mutated or point-mutated gene of interest was used for cassette exchange by homologous recombination.

2.1.7 Isolation of genomic DNA from *S. pombe*

A single colony was grown overnight to saturation in 10 ml of YE medium at 30°C with shaking. The pellet was resuspended in 2 ml SP1 buffer pH 5.6 (1.2 M sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate pH 5.6, 40 mM EDTA) and 1 mg/ml of zymolase. After incubation at 37°C for 45 minutes, the harvested pellet was resuspended in 900 µl of 5x TE buffer (50 mM Tris pH 8.0, 5 mM EDTA), followed by 100 µl of 10% SDS for 5 minutes and then 300 µl of 5 M potassium acetate. Samples were then incubated for 10 minutes on ice. Tubes were centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred to a new 1.5 ml tube containing an equal volume of isopropanol. The pellet was obtained by centrifugation at 13,000 rpm for 5 minutes and then washed with 500 µl of 70% ethanol and dried. The DNA was subsequently purified using a mixture of phenol-chloroform, followed by ethanol precipitation (Sambrook and Russell, 2001).

2.1.8 Crossing and random spore analysis

A loop of *S. pombe* cells of two different mating types was mixed with 10 µl of sterile water and the solution was then dropped on an ELN plate. The plate was incubated at 30°C for 3–5 days. The crossed cells were verified using a light microscope by checking for the presence of tetrads. A loop of the crossed cells was inoculated in 1 ml of sterile water containing 2 µl of Helicase (snail *Helix pomatia* juice) to digest the ascus wall and the solution were then incubated at 37°C overnight. The following day, the sample was diluted to reach the dilution of 10⁻² and 10⁻³. 100 µl of each dilution was spread onto YEA plates. All plates were incubated at 30°C for 3–5 days (Bähler *et al.*, 1998).

2.1.9 Spot test

After yeast strains were inoculated into YE liquid medium and grown at 30°C overnight in a shaking incubator, the cultures were diluted in the same medium to an optical density (OD) at 595 nm of approximately 0.2 and reincubated at the same conditions for 3 hours to ensure that the cells were in mid-log phase. The OD of these cultures was measured again (the cultures were re-diluted if required to ensure that all cultures were at the same OD). Ten-fold serial dilutions were made in order to produce 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions. From each one of these dilutions, 10 µl of each was spotted onto plates containing the different growth media such as YE agar, YNBA agar and YNBA agar containing rapamycin and/or NH_4Cl . All plates were then incubated at 30°C for three days.

2.1.10 Polysome analysis

To extract polyribosome from *S. pombe*, it was first necessary to prepare 10–60% sucrose gradients in buffer A (20 mM Tris-HCl pH7.5, 50 mM KCl, 1 mM DTT, 10 mM MgCl_2) containing 100 µg/ml cycloheximide or in buffer B (20 mM Tris-HCl pH7.5, 50 mM KCl, 1 mM DTT) were prepared in 15-ml polycarbonate tubes. The gradient tubes were prepared a day before use by adding 500 µL of 60% sucrose solution. The tubes were then frozen in liquid nitrogen for 10 seconds. 1.6 ml of each other concentrations of sucrose solution added on top the higher concentration which was frozen in liquid nitrogen. The lowest sucrose concentration was added on top. Finally, the tubes were covered with parafilm and then stored at 4°C to thaw slowly overnight.

S. pombe cells were grown at 30°C until they were at OD 0.5. Cells were untreated and treated with cycloheximide at the final concentration of 0.1 mg/ml for 10 minutes at 30°C. Cycloheximide-treated cells were harvested and then resuspended with 0.5 ml lysis buffer (20 mM Tris-HCl pH 7.5, 140 mM KCl, 10 mM MgCl_2 , 1 mM DTT, 0.1 mg/ml cycloheximide, 0.2 mg/ml heparin, 1% Triton X-100, EDTA-free protease inhibitor (Roche)). Untreated cells were also harvested and resuspended with the same lysis buffer without cycloheximide but containing 2.5 mM EDTA. Cells were lysed by the sonication. The supernatant was separated by centrifugation at 13,000 rpm for 5 minutes at 4°C. 400 µl of each sample was loaded onto the corresponding sucrose gradient tubes. The tubes were centrifuged at 38,000 rpm for 130 minutes at 4°C (Beckman SW 40Ti rotor). The gradients were then fractionated by upward displacement with 65% sucrose to collect

each fraction using a fraction collector. RNA of each sample was measured by the OD260 reading.

2.2 Bacterial methods

2.2.1 Media

L-Broth (LB)

10 g/l tryptone

5 g/l yeast extract

10 g/l sodium chloride

8 g/l agar (for making a solid LB media only)

2.2.2 Antibiotics

Bacterial plasmids normally contain one or more antibiotic resistance markers. To select bacteria containing particular plasmids, antibiotics were added to media prior to use. All antibiotics were kept at -20°C . There were three antibiotics used in this study, namely ampicillin (100 $\mu\text{g/ml}$ final concentration), kanamycin (100 $\mu\text{g/ml}$ final concentration) and chloramphenicol (50 $\mu\text{g/ml}$ final concentration).

2.2.3 Bacterial strains

There were two strains of *Escherichia coli* (*E. coli*) that were used in this study.

1) NM522, *supE*, *thi-1*, $\Delta(\text{lac-proAB})$, *hsd5* (r^{-} , m^{-}), [F' , *proAB*, *lacI^qZ Δ M15*]

2) BL21 (DE3), pLysS, F^{-} , *ompT*, *hsdSB* (rB^{-} , mB^{-}), *dcm*, *gal*, $\lambda(\text{DE3})$, pLysS, *Cm^r*

2.2.4 Bacterial cloning vectors

There are many bacterial cloning vectors used in this study (Table 2.2), namely pGEM-T Easy (Promega), pET15b (Novagen), pET28a (Novagen), pET28b (Novagen), pGEX (Pharmacia) and pCMV6 (OriGene).

Table 2.2 Bacterial cloning vectors used in this study.

Vector	Promoter	Tag	Selection
pGEM-T Easy	T7, SP6	-	<i>Amp^r</i>
pET15b	T7lac	N-terminal 6xHis	<i>Amp^r</i>
pET28a	T7lac	N-terminal 6xHis	<i>Kan^r</i>
pET28b	T7lac	N-terminal 6xHis	<i>Kan^r</i>
pGEX	tac (trp/lac)	N-terminal GST	<i>Amp^r</i>
pCMV6	CMV	C-terminal myc-Flag	<i>Kan^r</i>

2.2.5 Competent cells

A single colony was grown overnight at 37°C in 5 ml LB (containing the appropriate antibiotics). The pre-culture was used to inoculate a larger scale culture in a one litre of pre-warmed LB and then was incubated at 37°C with shaking for 2–4 hours until OD₆₀₀ ~ 0.5–0.6. The large culture was chilled on ice for at least half an hour and then centrifuged using SLA3000 rotor at 5,000 rpm for 5 minutes at 4°C. The supernatant was discarded. The pellet was resuspended in 25 ml of ice-cold TRNS1 solution (100 mM rubidium chloride, 50 mM manganese (II) chloride, 10 mM calcium chloride, 35 mM sodium acetate, 6.6% glycerol, pH 5.8 was adjusted by 0.2 M acetic acid) and then was incubated on ice for an hour. The cells were harvested by centrifugation at 5,000 rpm for 5 minutes at 4°C. The pellet was resuspended in 12 ml of TRNS2 solution (10 mM rubidium chloride, 75 mM calcium chloride, 10 mM MOPS, 6.6% glycerol, pH 6.8 adjusted with potassium hydroxide). The cell suspension was incubated on ice for an hour. 300 µl of the cell suspension was aliquoted into tubes and frozen in liquid nitrogen. Competent cells were stored at –80°C.

2.2.6 Bacterial transformation

The competent cells, *E. coli* NM522 or BL21, were thawed on ice. Approximately 20 ng (1 µl) of plasmid or ligation products was added to tubes containing 100 µl of the cells. The mixture was incubated on ice for 20 minutes. Heat shock was performed at 37°C for 90 seconds. 1 ml of LB medium was directly added to the tubes. The tubes were incubated at 37°C for 1 hour. Cells were harvested by centrifugation and the pellet was

resuspended with 100 μ l of remaining supernatant. The mixture was placed onto LB agar plates with specific antibiotic agents to select for transformed cells.

2.2.7 White-blue selection

This technique is a screening method which is used to screen ligations in vector-based gene cloning. This method needs 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-Gal) which is a substrate of the enzyme, β -galactosidase encoded by the *lacZ* gene of the *lac* operon. In this study, NM522 was normally used as it contains *lacI^q* on an F'episome. The transformed cells were screened on a LB agar plate containing 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and 20 μ g/ml X-Gal in DMSO or dimethylformamide (DMF). The stock solutions of 0.1 M IPTG and 40 mg/ml X-Gal were stored at -20°C . Transformed cells containing non-recombinant vectors will produce blue colonies, while cells containing recombinant vectors will form white colonies. The white colonies, however, will be further analysed in order to confirm the appropriate sequences using other techniques e.g. digestion with restriction enzymes or sequencing.

2.3 DNA methods

2.3.1 Plasmid DNA Preparation

A single transformed colony was inoculated and grown in 10 ml of LB medium with appropriate antibiotics at 37°C . Plasmid DNA was isolated using a QIAprep® Miniprep kit (QIAGEN) according to the manufacturer's instructions.

2.3.2 Polymerase chain reaction

The reaction mixture was generally set up in a final volume of 100 μ l. The PCR mixture contained 0.2–10 ng plasmid DNA or 20–100 ng of yeast genomic DNA or cDNA, 10 μ l of 10x ThermoPol Reaction Buffer (NEB), 10 mM of each dNTP, 10 μ M of each oligonucleotide primer, 2.5 units of a thermostable *Pfu* DNA Polymerase and adjusted with water up to 100 μ l. To amplify DNA sequences, the PCR tubes were placed on a thermal cycler machine. The PCR was heated to 94°C for 2 minutes. The cycler was programmed to cycle between three different temperatures. Typically, 20–25 cycles of 94°C for 30 second, an appropriate annealing temperature for 1 minute and 68°C for 1–

2 minutes per a kilobase was performed. The reaction was subsequently heated at 68°C for another 10 minutes and kept at 4°C.

2.3.3 DNA digestion with restriction enzymes

The reactions for DNA digestion were performed using a final concentration of 1x enzyme buffer, 1–20 µg DNA and 1 µl of each of restriction enzymes as well as water up to total volume of 50 µl. The reactions were incubated at 37°C for an hour or as recommended by the manufacturer. The insert DNA fragments were then purified from an agarose gel. In the case of vectors requiring de-phosphorylation, 10 units of calf intestine alkaline phosphatase (CIP) was added directly into the reaction for 10 minutes at 37°C, prior to DNA purification by QIAquick PCR purification kit (QIAGEN).

2.3.4 Agarose gel electrophoresis

To make a 0.8% agarose gel, 2.4 g agarose was added to 300 ml of 1x TBE buffer (89 mM Tris base, 2.5 mM EDTA, 89 mM g/l boric acid). The mixture was microwaved for 1 minute and mixed gently (these steps were repeated until the agarose had dissolved). The agarose was cooled for 30–60 seconds at room temperature. 5 µl ethidium bromide was added to the solution, and swirled gently. The solution was poured into a gel cast containing a gel comb. The gel was allowed to polymerize for 30 minutes at room temperature. To load samples, the comb was removed, and then the agarose gel was placed in a gel box containing 1x TBE buffer. Samples were mixed with 6x loading buffer (0.25% bromophenol blue, 15% Ficoll-Type 400) and loaded into wells. The sample on the gel was run at the voltage of 100–150 V for 30–45 minutes.

2.3.5 Purification of DNA from agarose gel using Easy Pure kit

The agarose gel slice containing the DNA fragment was dissolved using 300 µl of SALT solution (a chaotropic salt) and 50 µl of MELT solution (guanidine thiocyanate) at 55°C for 5–10 minutes. 8 µl of bead matrix were added to bind the DNA fragments. The contaminants were removed by washing with 1 ml of Easy Pure Wash solution. The purified DNA fragments were eluted into water. The eluted DNA was ready to use for ligation.

2.3.6 Ligation reaction

DNA ligation was typically carried out in a final volume of 20 μ l. The reaction was set up with 10 μ l of 2x Quick ligation reaction buffer, 50 ng of the linear vector, which had been treated with the restriction enzymes and alkaline phosphatase, a three-fold molar ratio of the insert DNA fragments and 1 μ l of Quick T4 DNA ligase (NEB) according to manufacturer's instructions. The reaction was mixed thoroughly and incubated at room temperature for 5 minutes.

2.3.7 Site-directed mutagenesis by PCR technique

Unlike the normal PCR technique, this procedure required a forward primer that was around 30 bases in length containing the mutant base(s) and a reverse primer that was complementary to the forward primer (Kunkel, 1985). The reaction mixture was normally set up in a total volume of 50 μ l. The PCR mixture contained 0.2–10 ng plasmid DNA, 5 μ l of 10x ThermoPol Reaction Buffer (NEB), 0.2–0.5 mM of each dNTP, 0.2–0.5 mM of each oligonucleotide primer, 2.5 units of a thermostable Pfu DNA polymerase and adjusted with water up to 50 μ l. The PCR tubes were then placed in a thermal cycler machine. The PCR was heated to 94°C for 2 minutes. 20–25 cycles were performed with denaturing temperature for 30–60 seconds, annealing temperature for 1 minute and 68°C (*Pfu* DNA Polymerase) for 1–2 minutes per a kilobase for extension. Subsequently, the mixture was heated at 68°C (*Pfu* DNA polymerase) for another 20 minutes and kept at 4°C. The product was digested with *DpnI* to cleave only at methylated sites of the template plasmid. The final reaction was transformed into competent NM522 cells. Plasmids were then extracted and sent for sequencing at Source BioScience or GATC.

2.4 Mammalian cell culture methods

2.4.1 Mammalian cell culture media, antibiotics and supplements.

- 1) Penicillin and streptomycin solution (Sigma-Aldrich) (100x)

10,000 units penicillin

10 mg/ml streptomycin

- 2) L-Glutamine solution (Sigma-Aldrich) (100x)

200 mM L-Glutamine

* These two solutions were frozen at –20°C. They were thawed in a water bath at 37°C for at least half an hour prior to use.

3) Fetal calf serum (FCS) (Gibco®, Life Technologies)

This serum was aliquoted and frozen at -20°C . It was thawed at 37°C for at least half an hour prior to use.

4) DMEM medium with high glucose (Gibco®, Life Technologies)

This 500-ml DMEM medium was supplemented with 5 ml of 200 mM L-glutamine (100x), 90 ml of FCS and 5 ml of 100x penicillin and streptomycin solution.

5) Coon's Modified Ham's F12 medium (Sigma-Aldrich, F6636)

Nutrient Mixture F-12 Coon's modification powder including 0.292 g/l L-glutamine and 0.863 mg/l zinc sulphate was added to a litre of water. 2.68 g/l sodium bicarbonate (NaHCO_3) was added in the solution. This medium was prepared according to the manufacturer's instructions (Sigma-Aldrich). Then 500 ml of the medium was supplemented with 100x penicillin and streptomycin solution and 60 ml of FCS.

2.4.2 Cell culture

HeLa, MRC5, MCF7, MDA-MB231 cells were cultured as monolayers in DMEM medium supplemented with 15% fetal calf serum (FCS) and incubated at 37°C with 5% CO_2 for 1–3 days to reach 70–80% confluence. PC3 cells were grown as a monolayer in Coons Modified Ham's F12 medium containing 2mM glutamine and 12% FCS and incubated at 37°C with 5% CO_2 for 1–3 days to reach 70–80% confluence. Details of these cells are shown in Table 2.3.

Table 2.3 Human cell lines used in this study.

Cell line	Organism	Origin	Cell type	Disease
HeLa*	human	cervix	epithelial	adenocarcinoma
MRC5	human	lung	fibroblast	fibroblast
MCF7	human	breast	epithelial	adenocarcinoma
MDA-MB231	human	breast	epithelial	adenocarcinoma
PC3	human	prostate	epithelial	adenocarcinoma

2.4.3 Trypsinization of adherent cells

To trypsinize the adherent cells, they needed to be at 70–80% confluence. The medium was aspirated off in a biohazard hood. The cells were washed twice with PBS. 0.25% trypsin solution was added. The flask or plate was incubated at 37°C with 5% CO₂ for 5 minutes for trypsin to work. One volume of warm medium was added and the mixture was transferred into a sterile tube. The tube was then centrifuged at 1,500 g for 5 minutes at room temperature. The supernatant was carefully discarded in the hood. The pellet was resuspended in an appropriate medium.

2.4.4 Freezing of mammalian cells

After cells were trypsinized, they were counted in a haemocytometer. They were then pelleted in a conical tube by centrifugation at 1,500 rpm for 5 minutes. The pellet was resuspended in freezing medium (10% DMSO, 90% completed media which containing FSC) to dilute to a final concentration of 1×10^6 cells/ml. 2 ml of the resuspension was aliquoted in each cryotube vial. The tube was kept in a thermal insulated box and this was placed in a –80°C for 24 hours. Then the vials was stored in a liquid nitrogen container.

2.4.5 Thawing of mammalian cells

A vial was taken from the liquid nitrogen container and thawed rapidly by swirling in a 37°C water bath. The outside of the tube was sterilized with IMS. Cell solution was dropped carefully onto the top of 10 ml of pre-warmed media containing 10% DMSO in a new conical tube. The tube was centrifuged at 1,500 rpm for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in the growth medium. The cells were grown in the same conditions as previously described.

2.4.6 Arsenite treatment

60–80% confluent cells were treated with 1 mM sodium arsenite and then incubated at 37°C for 30 minutes.

2.4.7 Radiation sensitivity test

Cells were irradiated using a [¹³⁷Cs] gamma source with a dose of 3 Gy, and then allowed to recover at 37°C for 30 minutes.

2.4.8 Immunofluorescence

1×10^5 mammalian cells were cultured in 2 ml of the appropriate media in a six-well plate containing cover slips in each well at 37°C for 48 hours. The cells needed to be at 60–80% confluent for treatment with any conditions. The supernatant was aspirated off and the cells were washed twice with PBS. The cells were fixed and permeabilized with cold absolute methanol for 20 minutes at –20°C. The cells were blocked with 2% BSA (bovine serum albumin) in PBS for 30 minutes at room temperature. The BSA solution was removed and replaced with appropriate dilution of primary antibody in 2% BSA. The primary antibodies were typically used at a 1:50 dilution in 2% BSA. From this step on, the cells were kept in a humid atmosphere to prevent them from drying out. The reaction was incubated for 1 hour at room temperature after that the cells were washed three times with PBS. The 1:200 dilution of specific secondary antibody in 2% BSA was then added. The reaction was incubated for at least 40 minutes at room temperature. The cells were washed three times with PBS. To mount the cells, 15 μ l of ProLong® Gold Antifade Reagent with DAPI was dropped on a slide and the coverslip containing the cells was mounted upside down and placed on top of the DAPI reagent. The reaction was incubated for an hour at room temperature. Slides were kept at 4°C prior to examination by wide field fluorescence microscopy (DeltaVision) according to the manufacturer's instructions.

Table 2.4 List of primary and secondary antibodies used in immunofluorescence assay

Antibody	Source	Type	Clone	Company	Dilution
anti-eIF4G	mouse	primary	monoclonal	Santa Cruz (sc-373892)	1:50
anti-eIF4E	rabbit	primary	polyclonal	In-house	1:100
anti-eIF4A	rabbit	primary	polyclonal	In-house	1:100
anti-SUMO1	rabbit	primary	polyclonal	Santa Cruz (sc-9060)	1:50
anti-SUMO1	mouse	primary	monoclonal	Santa Cruz (sc-5308)	1:50
FITC-anti-mouse IgG	goat	secondary	polyclonal	Sigma (F0257)	1:200
Cy3-anti-rabbit IgG	sheep	secondary	polyclonal	Sigma (C2306)	1:200

2.4.9 Two-step transfection of siRNA and plasmids

A mixture of siRNA was prepared in a 1.5-ml tube by the addition of 50 μ l Opti-MEM (31985-047, Life Technologies), 5 μ l HiPerFect transfection reagent (301705, QIAGEN) and 10–30 nM siRNA eIF4AII (s4572, Life Technologies). The siRNA mixture was incubated at room temperature for 10 minutes. 2×10^5 HeLa cells were added in 2 ml DMEM in a six-well plate, followed by the addition of the siRNA mixture, then incubated at 37°C for 48–72 hours. The cells needed to be at approximately 70% confluent. The cells were washed twice by PBS, and 2 ml fresh medium was added into each well. A mixture of plasmid was prepared by the addition of 130 μ l Opti-MEM, 6 μ l FuGENE® HD transfection reagent (e2311, Promega) and 2–3 μ g plasmid DNA, then incubated at room temperature for 10 minutes. The plasmid mixture was added into the six-well plate. This was incubated at 37°C for 24 hours. The cells were harvested for further analysis.

2.5 Protein methods

2.5.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was carried out using Bio-Rad Mini Protean II apparatus. Protein samples were resolved on the various percentage of separating gels, commonly 7.5–12.5%. Separating gels were prepared by mixing protogel (30% acrylamide: 0.8% bisacrylamide) and 4x separating buffer which was polymerised by the addition of 10% ammonium persulphate (APS) and TEMED (N,N,N',N'-tertamethylethylenediamine). The volume added was shown in Table 2.5. The pre-polymerised mixture was poured in the 1-mm gap between two glass plates and allowed to set for 30–45 minutes with a layer of isopropanol on top to get a level surface. After the gel was set, the isopropanol layer was washed off with distilled water. The stacking gel was prepared (usually at 3%) (Table 2.6), and then poured on top of the separating gel. The gel comb was positioned. After the stacking gel was allowed to set for 30 minutes, the comb was removed and the gel kit was then assembled. Protein samples were mixed with 5x SDS sample buffer and denatured at 95°C for 5–10 minutes. 10–30 µl of protein samples were loaded into each well. 8 µl of ColorPlus™ Prestained Protein Ladder (New England Biolabs) was loaded into a lane as a size indicator. Gels were run in 1x SDS running buffer at 150 V for approximately 1 hour or until the dye front reached the bottom of the gel.

Table 2.5 Separating gel formulation for preparing 2 gels

Separating Gel (2 gels)	7.5%	10.0%	12.5%
30% Protogel (ml)	2.5	3.3	4.2
4x separating buffer (ml) (1.5 M Tris-HCl pH 8.8, 0.4% SDS)	2.5	2.5	2.5
Distilled water (ml)	5.0	4.2	3.3
10% APS (µl)	100	100	100
TEMED (µl)	10	10	10

Table 2.6 Stacking gel formulation for preparing 2 gels

Stacking Gel (2 gels)	3%	6%
30% Protogel (ml)	0.5	1.0
4x stacking buffer (ml) (0.5 M Tris-HCl pH 6.8, 0.4% SDS)	1.3	1.3
Distilled water (ml)	3.3	2.8
10% APS (μ l)	50	50
TEMED (μ l)	10	10

5x Sample buffer:

60 mM	Tris HCl, pH 6.8
25%	glycerol
2%	SDS
14.4 mM	β -mercaptoethanol
10%	Bromophenol blue

10x SDS-PAGE buffer:

25 mM	Tris HCl, pH 8.3
192 mM	glycine
0.1%	SDS

2.5.2 Coomassie staining

An SDS-PAGE gel was placed in Coomassie gel stain (1 g/l Coomassie Brilliant Blue (Sigma), 45% methanol, 10% glacial acetic acid) at room temperature for 1 hour with gentle shaking. The gel was then briefly washed in water and then placed in destain solution (10% methanol, 10% glacial acetic acid) overnight with gentle shaking. To dry the SDS-PAGE gel, the gel was placed on Whatman 3 MM paper and dried for 1 hour on a gel dryer.

2.5.3 Whole cell extraction

3–5 $\times 10^5$ yeast cells or 5–10 $\times 10^5$ mammalian cells were washed once with pre-cold PBS. The cell pellet was resuspended with 500 μ l of ice-cold water, and then 75 μ l of lysis solution was added (1.85 NaOH, 7.5%v/v β -mercaptoethanol). The mixture was incubated on ice for 15 minutes. Then 75 μ l of 50% TCA was added into the lysate tube,

and the tube was reincubated on ice for 10 minutes. Denatured proteins were precipitated by centrifugation at 13,000 g for 20 minutes at 4°C. The supernatant was aspirated off. The tube was re-centrifuged for 1 minute and the remaining supernatant was removed. The precipitate was resuspended with 30 µl of 1xSDS sample buffer [if it turned yellow, 1 µl neutralising buffer (1.5 M Tris-HCl pH 8.8) was added until the sample turned blue]. The sample was heated to denature proteins at 95°C for 5–10 minutes. The supernatant was collected by centrifugation at 13,000 rpm for 5 minutes. The sample was stored at –20°C.

2.5.4 Western blotting

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore). Twelve pieces of Whatman 3MM papers were soaked in 1x Semi-dry transfer buffer (48 mM Tris base, 39 mM glycine, 0.04% SDS, 20% methanol). The first 6 pieces of the papers were placed on a semi-dry electroblotter (Biorad). The PVDF membrane soaked in methanol was placed on top of the 6 Whatman sheets. The protein gel was laid on top of the membrane and the remaining papers placed on top. Bubbles were removed by rolling a glass tube over the stack. The electroblotter was run at 150 mA for 35 minutes per gel. Following blotting the PVDF membrane was transferred to a container containing 4% milk (in PBS) and was blocked for at least 30 minutes at room temperature with gentle shaking.

2.5.5 Antibodies used for immunoblotting technique

Primary and secondary antibodies were required for immunoblotting method (Table 2.7). There were several primary antibodies which were specifically used for probing the protein of interest, namely specific rabbit anti-eIF4G antisera, rabbit anti-eIF4A antisera, rabbit anti-eIF4E antisera (kindly provided by Prof Simon Morley), mouse monoclonal eIF4G antibody, rabbit polyclonal SUMO1 antibody, rabbit polyclonal SUMO 2+3 antibody, mouse monoclonal anti-HA antibody, mouse monoclonal anti-c-myc antibody, rabbit polyclonal β -tubulin antibody (Santa Cruz Biotechnology), mouse monoclonal anti-FLAG antibody (Sigma) and rabbit polyclonal anti-eIF4AII antibody (abcam). For secondary antibodies, there were two types which were used in this study, namely polyclonal goat anti-rabbit immunoglobulins/HRP (Dako) and polyclonal rabbit anti-Mouse immunoglobulins/HRP (Dako).

Table 2.7 List of primary and secondary antibodies used in immunoblotting technique

Antibody	Source	Type	Clone	Company	Dilution
anti-eIF4G	rabbit	primary	polyclonal	In-house	1:10,000
anti-eIF4G	mouse	primary	monoclonal	Santa Cruz (sc-373892)	1:2,500
anti-eIF4E	rabbit	primary	polyclonal	In-house	1:3,000
anti-eIF4A	rabbit	primary	polyclonal	In-house	1:3,000
anti-eIF4AII	rabbit	primary	polyclonal	Abcam (ab31218)	1:3,000
anti-SUMO	rabbit	primary	polyclonal	In-house	1:2,500
anti-SUMO1	rabbit	primary	polyclonal	Santa Cruz (sc-9060)	1:2,500
anti-SUMO1	mouse	primary	monoclonal	Santa Cruz (sc-5308)	1:2,500
anti-SUMO2/3	rabbit	primary	polyclonal	Santa Cruz (sc-32873)	1:2,500
anti-c-Myc	mouse	primary	monoclonal	Santa Cruz (sc-40)	1:2,500
anti-HA	mouse	primary	monoclonal	Santa Cruz (sc-7392)	1:2,500
anti-FLAG	mouse	primary	monoclonal	Sigma (F1804)	1:1,000
anti- β -tubulin	rabbit	primary	polyclonal	Santa Cruz (sc-9107)	1:2,500

2.5.6 PVDF membrane incubated with antibodies

After the PVDF membrane was blocked in 4% milk (in PBS), the membrane was then incubated in 5–10 ml milk containing the primary antibody. The primary antibodies were typically used at a 1:2,500 dilution in 4% milk (in PBS) and incubated overnight at 4°C with gentle shaking. Alterations to this general protocol were made depending on the efficiency of the particular antibody (Table 2.7). The membrane was washed three times with PBS containing 0.1% Tween-20 (PBST) for 10 minutes each wash. After that 10 ml of 4% milk (in PBS) was added to the membrane and an HRP-conjugated secondary antibody was added to a final dilution of 1:2,500 (Table 2.7). The blot was incubated at room temperature for 1–2 hours with gentle shaking. The membrane was washed twice with PBST for 5–10 minutes, and another wash with PBS 5–10 minutes, respectively.

2.5.7 Chemi-Luminescent Detection

The proteins on the PVDF membrane were detected by ECL enhanced chemiluminescence. Detection was carried out using ECL Western Blotting substrate (Thermo Scientific). The blot was incubated for 1 minute with slight agitation. Then the blot was removed onto a transparency wrap or a transparency sheet and then placed in a film cassette. In a dark room, the membrane was exposed to X-ray film for varying lengths of time depending on the intensity of the signal. The film was developed using a LSC50000 machine.

2.5.8 Bradford assay

To determine the protein concentration of a sample the Bradford assay reagent (Biorad) was diluted 1 in 5 with water. 1 µl protein sample was added to 1 ml of the diluted Bradford reagent. The OD595 was measured as compared to a 1 ml reagent only 'blank'. The protein concentration of the sample was determined by comparing the sample reading against a BSA standard curve.

2.5.9 *In vitro* SUMOylation assay

N-terminally His-tagged Pmt3-GG protein, a C-terminally truncated version of Pmt3 and other SUMOylation components were purified as described by Ho *et al* (Ho *et al.*, 2001). The *in vitro* SUMO modification assay was performed in 50 mM Tris-HCl pH7.5, 5 mM MgCl₂, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase,

0.6 U/ml inorganic pyrophosphatase using 3 µg GST-Hus5, 8–10 µg His-SUMO-GG or His-SUMO-Tr-GG, 0.4–0.6 µg SAE1 (His-Rad31 and GST-Fub2) and 6.6–15 µg protein target. 1 µg His-Pli1 was added to the reaction as optional. The reactions were incubated at 30°C for 2 hours and analysed by SDS-PAGE.

2.5.10 In-solution digestion

Proteins in any solution were diluted in 25–50 mM ammonium bicarbonate to a final concentration of up to 1 mg/ml. Proteins were reduced in 10 mM dithiothreitol, 25 mM ammonium bicarbonate for 1 hour at 56°C and then alkylated in 55 mM iodoacetamide, 25 mM ammonium bicarbonate for 45 minutes at room temperature in the dark. Mass spectrometry-grade trypsin (Promega, WI, USA) was added at a ratio of 1:50 (weight of trypsin: weight of protein) and hydrolysis was allowed to occur at 37°C overnight. The reaction was quenched by addition of 0.5% trifluoroacetic acid. Peptides were concentrated using SpeedVac® concentrator (Thermo scientific, USA) according to the manufacturer's instructions.

2.5.11 In-gel digestion

Bands from polyacrylamide gels were excised and divided into 4–5 small pieces. Coomassie-stained bands were destained twice with 50% acetonitrile, 25 mM ammonium bicarbonate for 15 minutes at room temperature with agitation, repeated if necessary until the gel pieces were destained. Destained gel pieces were dried using vacuum centrifugation for 5 minutes. Gel pieces were then rehydrated in 10 mM dithiothreitol, 25 mM ammonium bicarbonate and reduced at 56°C for 45 minutes. The liquid was removed and replaced with 55 mM iodoacetamide, 25 mM ammonium bicarbonate. Gel pieces were alkylated for 45 minutes at room temperature in the dark. The supernatant was removed and gel pieces were dehydrated twice with 50% acetonitrile, 25 mM ammonium bicarbonate for 5 minutes at room temperature with agitation. 25 µg of Mass spectrometry-grade trypsin (Promega, WI, USA) was resuspended in 25 µl of 50 mM acetic acid resuspension buffer (Promega) and incubated at 30°C for 15 minutes. Trypsin solution was then diluted in 25 mM ammonium bicarbonate to give a 25 ng/µl solution. Gel pieces were dried and rehydrated in 25 ng/µl trypsin, 25 mM ammonium bicarbonate. Excess trypsin solution was removed after 10 minutes, the gel pieces were covered with 25 mM ammonium bicarbonate and hydrolysis was allowed to occur overnight at 37°C. Trifluoroacetic acid was added to 0.5% and the supernatant was

removed to a clean tube. Remaining peptides were extracted from the gel pieces using 50% acetonitrile with vigorous agitation, repeated if gel pieces were not dehydrated. All supernatants were pooled and the volume was reduced using SpeedVac® concentrator (Thermo scientific, USA).

2.5.12 Protein expression from BL21 (DE3) pLysS *E. coli* cells

An expression plasmid containing the gene encoding the protein of interest was transformed into BL21 (DE3) pLysS *E. coli* cells, following the bacterial transformation protocol (see section 2.2.6). Cells were plated on LA plates containing chloramphenicol and the appropriate antibiotics, and then grown overnight at 37°C. A single colony was inoculated in 10 ml liquid culture with antibiotic selection and grown overnight at 37°C with shaking. The culture was added to one litre of liquid medium with antibiotics and incubated at 37°C until the OD600 reached between 0.4 and 0.8. Expression of the protein of interest was induced with IPTG at a final concentration of 0.1–0.4 mM (0.15 mM, commonly used in this study) overnight at 16°C with shaking. The cell pellet was harvested by centrifugation at 5,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was scraped into a pre-cooled 50-ml tube. A loop of cells was collected to check protein expression using either a Coomassie-stained protein gel, western blotting or an activity assay. The rest of the pellet was frozen using liquid nitrogen and stored at –80°C overnight or until needed.

2.5.13 His-tagged protein purification by Ni agarose beads under native buffer condition

A single colony of BL21 (DE3) *E. coli* cells containing His-tagged expression vector was grown overnight at 37°C in L-broth containing chloramphenicol and the selective antibiotic. IPTG was added to induce protein expression (see section 2.5.12). The cell pellet was resuspended in 15 ml ice-cold Ni²⁺ binding buffer (20 mM Tris-HCl pH 7.9, 300 mM NaCl, 5 mM imidazole) containing protease inhibitors (Roche) and 0.1 mM PMSF. The cells were lysed on ice by sonication at 27% amplitude for 3 minutes (5 seconds on and 5 seconds off). The cell debris was removed by centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatant was applied to a 5-ml column containing 0.5 ml of Ni²⁺-agarose beads equilibrated with Ni²⁺ binding buffer at 4°C. After all of the supernatant flowed through the column by gravity, 10 ml (2x 5 ml) binding buffer was applied to the column. To wash the beads, 6 ml of wash buffer (20 mM Tris-HCl pH 7.9,

300 mM NaCl, 20 mM imidazole) was added to the column. To elute His-tagged fusion protein, 0.3 ml of elution buffer (20 mM Tris HCl pH 7.9, 300 mM NaCl, 250 mM imidazole) was added to the column (normally repeated 5 times). Each fraction was collected on ice and measured the protein concentration by Bradford assay. The His-tagged protein was also analysed by SDS-PAGE. Each protein elution was added with glycerol at the 10% final concentration in order to store at -80°C

2.5.14 GST-tagged protein purification under native buffer condition

A single colony of BL21 (DE3) *E. coli* cells containing GST-tagged expression vector was grown overnight at 37°C in L-broth containing chloramphenicol and the selective antibiotic. IPTG was added to induce protein expression (see section 2.5.12). The cell pellet was resuspended in 15 ml ice-cold NETN binding buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.5% NP-40) containing protease inhibitors (Roche) and 0.1 mM PMSF. The cells were lysed on ice by the sonication at 27% amplitude for 3 minutes (5 seconds on and 5 seconds). The cell debris was removed by centrifugation at 15,000 rpm for 30 minutes at 4°C . The supernatant was applied to a 5-ml column containing 0.5 ml of GST beads equilibrated with NETN binding buffer at 4°C . After all of the supernatant flowed through the column by gravity, 5 ml of binding buffer was applied to the column. To wash the beads, 6 ml of wash buffer (120 mM NaCl, 100 mM Tris-HCl pH 8.0) was added to the column. To elute GST-tagged protein, 0.3 ml of elution buffer (120 mM NaCl, 100 mM Tris-HCl pH 8.0, 1 mM PMSF, 20 mM glutathione) was added to the column (normally repeated 5 times). Each fraction was collected on ice and measured the protein concentration by Bradford assay. The GST-tagged fusion protein was also analysed by SDS-PAGE. Each protein elution was added with 30 μl of 100% glycerol and then kept at -80°C .

2.5.15 Protein expression from *Sf9* insect cells using Baculovirus expression system

To amplify the P1 recombinant virus, *Sf9* insect cells, were diluted in a 35-mm petri dish with SF900IISFM medium without antibiotics at a cell density of 1×10^6 cells/ml. They were then incubated at 27°C for an hour to adhere to the bottom of the plate, and then transfected by adding 2 ml of the P1 Baculovirus under humid conditions at 27°C for 3–5 days. Transfected insect cells were monitored every 24 hours to check for contamination and for the number of insect cells infected. 5 days after transfection, 2 ml

of supernatant was transferred to sterile cryotubes containing 1% heated-inactivated FCS. The cryotubes were stored at 4°C in the dark as P2 recombinant virus. Cells were kept and whole cell extracts were prepared to check protein expression levels by western blotting, and compared with non-transfected cells. P2 recombinant Baculovirus was amplified the same ways as above but a six-well plate was replaced to achieve a higher amount of P3 recombinant Baculovirus. P4 and P5 recombinant Baculovirus was scaled up and collected, respectively.

Plaque assays were performed to purify virus and determine the viral titre (pfu/ml). The P5 viral stock was diluted from 10^{-5} to 10^{-7} . 100 µl of each viral dilution was added into their respective plates containing adherent *Sf9* insect cells in SF900IISFM at the cell density of 5×10^5 cells/ml and then each plate was incubated at room temperature for 1 hour. Liquid medium was pipetted off and replaced with 2 ml of 1% agarose containing medium. After the agarose had set, 1 ml of medium was added on top of the agarose in each plate. Plates were incubated at 27°C for 3–4 days. Cells were stained by adding 1 ml of 0.02% neutral red (in PBS) in each plate. Plates were incubated at 27°C in the dark for 2 hours. All liquid was pipetted off and plates were inverted overnight at room temperature in the dark. Plaques were counted for zones of clearing generated by infection of *Sf9* insect cells. The number of plaques was calculated as a viral titre.

Efficient protein expression using the Baculovirus system depends on the titre of virus, the multiplicity of infection (MOI) and the cell density. These were tested to optimize the appropriate volume of recombinant virus used in transfection. To express recombinant gene products, a cell density of 1×10^6 to 2×10^6 cells/ml and MOIs of 0.5 to 10 were commonly employed. The optimized viral volume was added to a 1000 ml flask containing insect cells at a cell density of 2×10^6 cells/ml and suspension medium. The flask was incubated at 27°C in a humidified incubator for 72 hours. Cells were harvested by centrifugation at 2,000 g for 10 minutes at 4°C. The pellet was kept at –80°C or used immediately.

2.5.16 Isolation of His/FLAG-eIF4G from Baculovirus-infected insect cells

After 72 hours, Baculovirus-infected insect cells were harvested at 4°C by centrifugation at 2,000 g for 10 minutes. The pellet was resuspended in 15 ml of Lysis Buffer A [40 mM MOPS (KOH) pH 7.2, 300 mM NaCl, 2 mM benzamidine, 20 mM

imidazole, 3.5 mM β -mercaptoethanol, 1% NP40 and protease inhibitor cocktail (Roche, Cat. no. 11873580001)]. Cells were sonicated on ice at 27% amplitude, 5 seconds on and 5 seconds off for 3 minutes and vortexed briefly. The cell lysate was left on ice for 10 minutes and centrifuged at 9,800 rpm in SS34 Sorvall rotor at 4°C for 20 minutes. The supernatant containing His/FLAG recombinant proteins was added to 1 ml of NTA-agarose, which was washed twice with Binding Buffer B [Lysis Buffer A without 1% NP40] in a 50 ml falcon tube. The tube was rotated at 4°C for at least 1 hour. The resin was pelleted at 2,000 g for 1 minute in a cooled centrifuge and washed twice with 25 ml of the Binding buffer B, followed twice by 25 ml of the Lysis Buffer A. The resin was then washed twice with Wash Buffer C [40 mM MOPS (KOH) pH 7.2, 500 mM NaCl, 2 mM benzamidine, 20 mM imidazole, 3.5 mM β -mercaptoethanol, 1% NP40 and protease inhibitor cocktail (Roche)] and washed twice with Binding Buffer B without protease inhibitors. The resin was transferred to an ice-cold 2 ml tube. Protein was eluted with 500 μ l of Elution Buffer D [40 mM MOPS (KOH) pH 7.2, 300 mM NaCl, 2 mM benzamidine, 250 mM imidazole, 3.5 mM β -mercaptoethanol] at 4°C on an end-over-end mixer for 15 minutes. Eluate was removed to a cold tube by centrifugation at 13,000 rpm for 1 minute and this was repeated 4 times. A Bradford assay was undertaken for each sample to check for protein concentration. Each elution was checked by either Coomassie-stained protein gel or western blotting. 50 μ l of 100% glycerol was added into sample tube containing high-yield proteins. The tubes were frozen and stored at -80°C.

2.5.17 Immunoprecipitation using anti-eIF4G antisera

Mammalian cells were harvested by trypsinization and washed with PBS. The cell pellet was lysed in 100 μ l IP Lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM sodium fluoride (NaF), 25 mM β -glycerophosphate, 0.1 mM sodium orthovanadate (Na_3VO_4), 0.2% Triton X-100, 0.3% NP-40, 10 mM N-ethylmaleimide, protease inhibitor cocktail (Roche)], and the lysate was then spun at 13,000 rpm for 5 minutes. The supernatant was recovered into a new 1.5 ml tube and the protein concentration was then measured by Bradford assay. For immunoprecipitation, 1 mg of lysate sample was diluted in 1 ml IP Lysis buffer and incubated with 40 μ g of anti-eIF4G antisera for 2 hours at 4°C. The immune complex were then incubated with 20 μ l of 50% washed Protein A beads with end-over-end mixing for 1 hour. The resin was washed three times

with 500 µl of IP lysis buffer. The recovered proteins were eluted with 2x SDS-PAGE sample buffer.

2.5.18 Isolation of eIF4F and associated factors using m⁷GTP-Sepharose 4B beads

Harvested cells were resuspended in 100 µl of MOPS Lysis buffer [20 mM MOPS pH 7.2, 75 mM KCl, 2 mM benzamidine, 7 mM β-mercaptoethanol, 25 mM NaF, 2 mM magnesium chloride (MgCl₂), 25 mM β-glycerophosphate, protease inhibitor cocktail (Roche)]. The supernatant was collected and transferred into a new 1.5 ml tube and protein concentration was then measured by Bradford assay. 30 µl of pre-washed 50% (v/v) m⁷GTP-Sepharose 4B (GE Healthcare) beads was added into tubes containing 100 µg of total protein. The samples were gently mixed, placed back on ice and repeated in every 5 minutes for 15 minutes. The resins were washed twice with 200 µl of MOPS lysis buffer. To elute the factors, the beads were resuspended with 20 µl SDS-PAGE sample buffer.

2.5.19 Ni²⁺-pulled down under denaturing conditions

This method is able to be used for both *S. pombe* and mammalian cells. For mammalian cells, cells were grown until 60–80% confluent were harvested and counted. 6–8 × 10⁶ cells were washed with pre-cold PBS. For *S. pombe* cells, cells were grown until they are at OD 0.5. 1 × 10⁹ cells were harvested and washed with pre-cold PBS. The cell pellet was resuspended with 5 ml of ice-cold water, and then 0.8 ml of lysis solution (1.85 NaOH, 7.5%v/v β-mercaptoethanol) was added. The mixture was incubated on ice for 20 minutes. 0.8 ml of 50% TCA was added into the tube containing the cell lysate, and the tube was incubated on ice for 20 minutes. Denatured proteins were precipitated by centrifugation at 8,000 g for 20 minutes at 4°C. The precipitate was resuspended with 1 ml of buffer A (10 mM Tris-HCl, 6 M guanidinium HCl, 0.1 M NaH₂PO₄, pH 8.0) and transferred into a new centrifuge tube. The precipitate was completely solubilised by rotating the tube on a wheel for an hour at room temperature. Cell debris was removed by centrifugation at 13,000 rpm for 10 minutes, and the supernatant was transferred into a new tube containing 30 µl of pre-washed Ni-NTA beads which was washed with buffer A and 0.05% Tween-20 three times. The mixture was supplemented with 5 µl of 10% Tween-20 and 15 µl of 1 M imidazole. The tube was incubated overnight at room temperature on a wheel. Beads were pelleted by centrifugation at 2,000 rpm for

2 minutes. The supernatant was pipetted off carefully. The bead pellet was washed twice with 1 ml of buffer A containing 0.05% Tween-20. The pellet was then washed twice with 1 ml of buffer B (10 mM Tris-HCl, 0.1 M NaH₂PO₄, pH 6.3, 0.05% Tween-20). The pellet was also washed twice with 1 ml of buffer B containing 40 mM imidazole. The supernatant was completely removed from the bead pellet. 30 µl of HU buffer (8 M urea, 200 mM Tris-HCl pH 6.8, 1 mM EDTA, 5% SDS, 0.1% bromophenol blue, 1.5% dithiothreitol) was added into the tube containing the beads. To denature proteins, the sample was heated at 95°C for 5–10 minutes. The supernatant was obtained by centrifugation at 13,000 rpm for 5 minutes. The sample was stored at -20°C prior to use.

A whole cell extract sample was needed as a control for the experiment. So 3×10^5 mammalian cells or 4×10^7 *S. pombe* cells were washed once with pre-cold PBS. The cell pellet was resuspended with 500 µl of ice-cold water, and then 75 µl of the same lysis solution was added. The mixture was incubated on ice for 15 minutes. Then 75 µl of 50% TCA was added into the lysate tube, and the tube was reincubated on ice for 10 minutes. Denatured proteins were precipitated by centrifugation at 13,000 g for 20 minutes at 4°C. The supernatant was aspirated off. The tube was re-centrifuged for 1 minutes and the remaining supernatant was removed. The precipitate was resuspended with 30 µl of HU buffer (if it turned yellow, 1 µl neutralising buffer (1.5 M Tris-HCl pH 8.8) was added until the sample turned blue. The sample was heated to denature proteins at 95°C for 5–10 minutes. The supernatant was collected by centrifugation at 13,000 rpm for 5 minutes. The sample was stored at -20°C.

2.5.20 Protein synthesis assay

50-60% confluent cells were incubated with [³⁵S]-methionine (MP Biomedicals, UK; 10 µCi/ml), then reincubated at 30°C. Cells were harvested and then washed with pre-cold PBS. Cells were lysed in 100 µl pre-cold lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate (Na₃VO₄), 0.2% Triton X-100, 0.3% NP-40, 10 mM N-ethylmaleimide, protease inhibitor cocktail (EDTA-free Roche), with the addition of 0.5% deoxycholate (DOC)] with vortexing. Cell debris was removed by centrifugation at 4°C for 5 minutes. Protein concentration was determined and recorded. 5 µl of supernatant was then spotted onto Whatman filter papers. Filter papers were soaked in 10% TCA containing 5 mM unlabelled L-methionine for 20 minutes, then boiled in 5% TCA. After the papers were

cooled at room temperature for 30–45 minute, they were washed in ethanol, then acetone, and air dried. Next the papers were placed in liquid scintillation fluid. Protein synthesis was measured using a scintillation counter and calculated as cpm/ μ g protein.

2.5.21 Identification of SUMOylation sites by mass spectrometry

Proteins were separated by SDS-PAGE. Gel slices were excised and proteins subjected to trypsin digestion (described in section 2.5.11). Peptide fractions were analysed by LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 nanoLC column to be separated using a gradient of acetonitrile. Data were analysed using the Human NCBI database in conjunction with the MASCOT search algorithm.

Chapter 3

Investigating the role of SUMOylation of *S. pombe* RNA binding proteins

3.1 Introduction

One of the studies in the Watts lab to identify SUMOylated proteins in *S. pombe* (described in section 1.9) involved affinity purification of TAP-tagged Ulp2 (SUMO protease) and associated proteins (Dr. Min Feng, University of Sussex). Interestingly, the TAP-Ulp2 co-purified with a number of proteins, many of which are involved in RNA metabolism, protein synthesis or RNA binding. These co-purified proteins included translation initiation factors and a number of other proteins e.g. eIF2 α , eIF3a, eIF3c, eIF3h, eIF4G, eEF1A2 and the La protein homolog (Sla1).

These preliminary data suggested a link between these proteins and SUMOylation. Moreover, a significant number of eukaryotic translation initiation factors (eIFs) in budding yeast (*S. cerevisiae*), plants (*A. thaliana*), insect cells (*D. melanogaster*) and mammalian cells (human and rat) have been identified in proteomic screens as being SUMOylated (Table 1.5). However, most proteins identified in these screens have not yet been confirmed as being modified by SUMO. This chapter will focus on determining whether the *S. pombe* La homolog protein and two eIFs, specifically eIF4G and eIF3h, are SUMOylated.

3.2 Background of the *S. pombe* La homolog protein (Sla1)

Sla1 is a nuclear RNA-binding phosphoprotein that is encoded by the *sla1* gene. It is the homologue of the human La gene. The *S. pombe* La protein, Sla1 contains conserved RNA recognition motifs (RRM1 and RRM2) (Tanabe *et al.*, 2003; Cherkasova *et al.*, 2012). The protein acts as a molecular chaperone by binding to newly synthesized transcripts of RNA polymerase III in order to protect the RNAs from 3' exonuclease during intranuclear maturation (Van Horn *et al.*, 1997; Cherkasova *et al.*, 2012). The La protein (Sla1) is non-essential in *S. pombe* and *S. cerevisiae* (Lhp1p), but essential in mammals (La). For example, *S. pombe* cells, where the *sla1* gene is replaced with the *ura4+* gene (*sla1::ura4+*), are able to grow in minimal medium without uracil, indicating that *sla1* is a

non-essential gene in *S. pombe* (Van Horn *et al.*, 1997). *S. pombe* cells with a *sla1* deletion (*sla1*Δ) grow slower than wild type cells in Edinburgh minimum medium (EMM), but grow relatively well in rich medium (YE). Briefly, a mutated human La protein, which lacks a motif required for pre-tRNA processing, when introduced into the *sla1*Δ cells does not rescue the slow growth phenotype (Intine *et al.*, 2000). However, it can be rescued by introducing either the wild type human, *S. cerevisiae* or *S. pombe* La protein into the cells, resulting in a functional pathway of tRNA maturation. This suggests that the *Sla1*-deleted *S. pombe* grows slowly because of a defect in tRNA metabolism (Van Horn *et al.*, 1997; Cherkasova *et al.*, 2012).

Ammonium chloride (NH₄Cl) in EMM is a major component that affects the low growth rate of the *sla1*-deleted cells. It has been shown that adding proline rather than NH₄Cl supports the growth of the *sla1*Δ cells in EMM, since proline is a poor nitrogen source (Weisman *et al.*, 2005; Weisman *et al.*, 2007). When NH₄Cl is added into YE medium, growth of the *sla1*-deleted cells is strongly inhibited. This indicates that *sla1*Δ cells are hypersensitive to NH₄Cl and may suggest that *Sla1* regulates nitrogen metabolism (Van Horn *et al.*, 1997). In addition, it is also found that *sla1*Δ cells are highly sensitive to rapamycin (Cherkasova *et al.*, 2012).

3.3 *Sla1* is endogenously modified by SUMO

In order to determine whether the La protein homolog (*Sla1*) is SUMOylated *in vivo*, SUMOylation of *Sla1* was investigated following expression of N-terminally HA-tagged *Sla1* from pREP41 (created by Dr. Felicity Watts) and 6xHis-tagged SUMO from pREP42 (Dr. Lauren Small, University of Sussex). Those plasmids were transformed into wild-type *S. pombe*, so that both HA-tagged *Sla1* and His-tagged SUMO were overexpressed. 6xHis-SUMO was pulled out using Ni²⁺ agarose beads under denaturing conditions (see 2.5.19). Figure 3.1 demonstrates that HA-tagged *Sla1* is specifically recovered in the presence of His-tagged SUMO (lane 1), but not recovered in the absence of His-tagged SUMO (lane 2). This indicates that *Sla1* is SUMOylated *in vivo* in *S. pombe*. This is consistent with the fact that the *S. pombe* La protein homolog interacts with SUMO protease Ulp2.

In addition, to investigate SUMOylation of *Sla1* expressed at endogenous levels, several attempts were undertaken to C-terminally tag the *S. pombe sla1* gene with *myc* and HA epitopes as well as GFP sequences since antibodies against *Sla1* were not available.

However the gene tagging experiments were unsuccessful, suggesting that addition of a C-terminal tag to Sla1 is lethal.

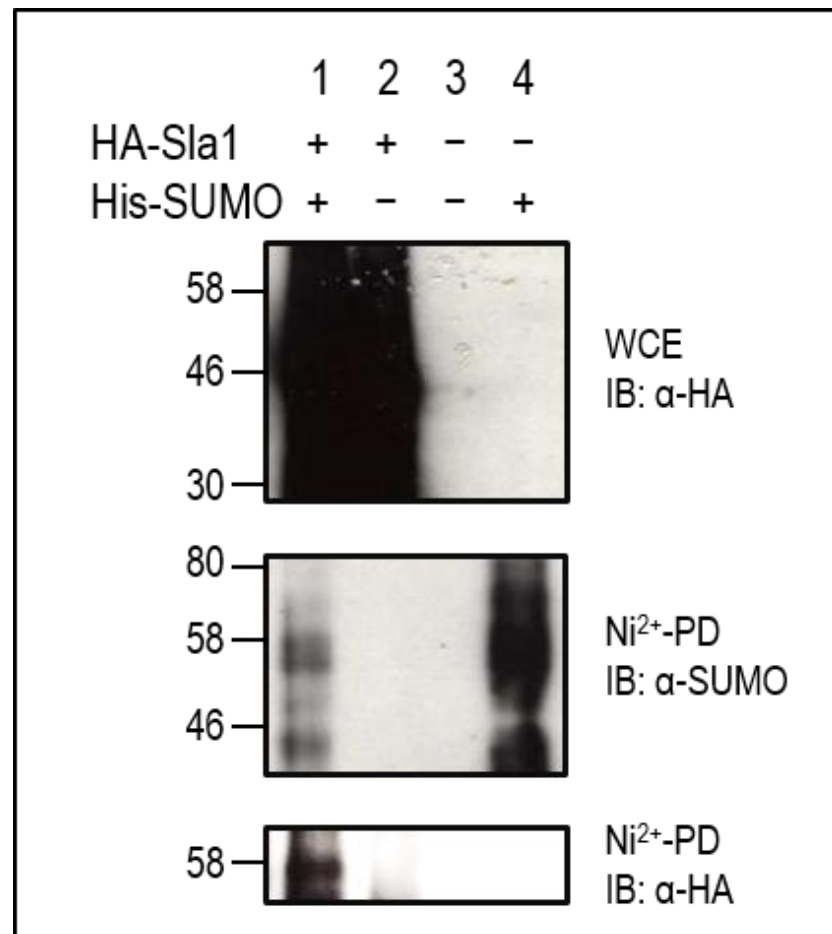


Figure 3.1 Western blotting of Ni²⁺ affinity purification for Sla1. His-tagged SUMO was affinity purified to test for SUMOylation of the La protein homolog (*sla1* product, Sla1). Whole cell extracts (WCE) and pulled down (Ni²⁺-PD) samples were analysed on 10% gels. Blots were probed with either anti-HA or anti-SUMO antisera.

3.4 Sla1 is SUMOylated *in vitro* on K106 and K263

Using an *in vitro* SUMOylation assay, Sla1 has previously been demonstrated to be modified by SUMO *in vitro* (P. Taylor, Watts lab). Mass spectrometric analysis of the modified form indicated that Sla1 is SUMOylated on lysines 106 and 263 (Dr. Brenda Mercer, University of Sussex).

3.5 Creating the SUMOylation mutants of La protein homolog from a *sla1* base strain

To investigate the role of SUMOylation of Sla1, strains containing unSUMOylatable versions of Sla1 were created. In order to do this, a *sla1* base strain was first created using the recombinase-mediated cassette exchange system for non-essential genes (Watson *et al.*, 2008). The PCR product, loxP-*ura4*⁺-loxM3, was amplified from the template pAW1 plasmids using a forward primer ‘Sla1 BS F’ and a reverse primer ‘Sla1 BS R’ (shown in Table 3.1) that consisted of ~80 bases homology to the upstream and downstream genomic target, respectively, plus 20 bases specific to pAW1. The PCR product was transformed into a *S. pombe* wild type strain to obtain the *sla1::ura4* base strain. To ensure that the integration was successful, genomic DNA was extracted and checked using two pairs of primers of ‘Sla1 external F’ and ‘Ura4 R’, another ‘Ura4 F’ and ‘Sla1 external R’ shown in Figure 3.2.

In parallel with the creation of a base strain, the chromosomal *sla1* gene along with its promoter and terminator was amplified using a pair of primers containing the sites for the restriction enzymes *Sph*I (Sla1 ORF Forward primer; 5’ GCATGCAGCAATCTCT GTTAATCTTTTCAAAAAAAAAAAC 3’) and *Sal*I (Sla1 ORF Reverse primer; 5’GTC GACATCCATTTTACTCCGAAGCGG 3’) at the 5’ end of the primers. These restriction sites are also present in the multiple cloning site (MCS) of pAW8 plasmid into which the PCR product was ligated.

To construct the plasmids containing the mutated residues of *sla1* SUMOylation sites, the pAW8-*sla1* plasmid was then used as PCR template. Site-directed mutagenesis was carried out on pAW8-*sla1* to mutate lysines at the positions 106 (K106) and 263 (K263) to arginine using primers ‘Sla 1 K106R F’ and ‘Sla1 K106 R’ thus creating pAW8-*sla1* K106R plasmid and ‘Sla 1 K263R F’ and ‘Sla 1 K263R R’ to create pAW8-*sla1* K263R plasmid. A doubly mutated plasmid (pAW8-*Sla1* K106R, K263R) was also constructed using the pAW8-*sla1* K106R plasmid as PCR template and the primers ‘Sla 1 K263R F’ and ‘Sla 1 K263R R’ instead. All of these plasmids were sequenced using a primer ‘Sla 1 Seq1 F’.

The wild-type and mutated pAW8-*sla1* plasmids were then used to transform the *sla1::ura4* base strain to achieve the *sla1.wt* strains (non-mutation), two strains containing a single lysine mutated to arginine (*sla1-K106R* and *sla1-K263R*) and one containing a

double mutation (*sla1-K106R,K263R* or *sla1-RR*) due to homologous recombination between the specific loxP and loxM sites (Figure 3.3).

Table 3.1 Primers involved in the construction of Sla1 strains.

Name	Primer sequences
Sla1 BS F	GTAAAGAATCATAAATAGGAAAAGCTAAAGCTACTGCTT GTCATTATGGTTAAATTGTATCCAGAGCTACATTGTAAGG CGGATCCCCGGGTTAATTAA
Sla BS R	CCTAATATAACCAATCCGAAAAAGTTTATTTGTCCAGTGT CTTTGAAGTATTTGTAAAAACAAACAAACAAAATTAG AATTCGAGCTCGTTTAAAC
Sla1 external F	GCTAGGAAATTTGAGGAAGTGGAG
Sla1 external R	GAAATGCAAGCTTGGTCGTACTGG
Ura4 F	TAGCGATATCATCATTTGTTGGTCGTG
Ura4 R	CCAATGTCTGCGAATTTGCGATCC
Sla1 ORF F	<u>GCATGC</u> AGCAATCTCTGTTAATCTTTTCAAAAAAAAAAAC <i>SphI</i>
Sla1 ORF R	<u>GTCGAC</u> ATCCATTTTACTCCGAAGCGG <i>SaII</i>
Sla 1 K106R F	CAAACAATTGCTAATTTTCAGACGTATGCGTCGTTTTC
Sla 1 K106R R	GAAAACGACGCATACGTCTGAAATTAGCAATTGTTTG
Sla 1 K263R F	CGTTTTGACGCTTTTAGAGAGATGGATCGTCAGC
Sla 1 K263R R	GCTGACGATCCATCTCTCTAAAAGCGTCAAAACG
Sla 1 Seq1 F	GGAAGCTGGAAAAGTAGTAG

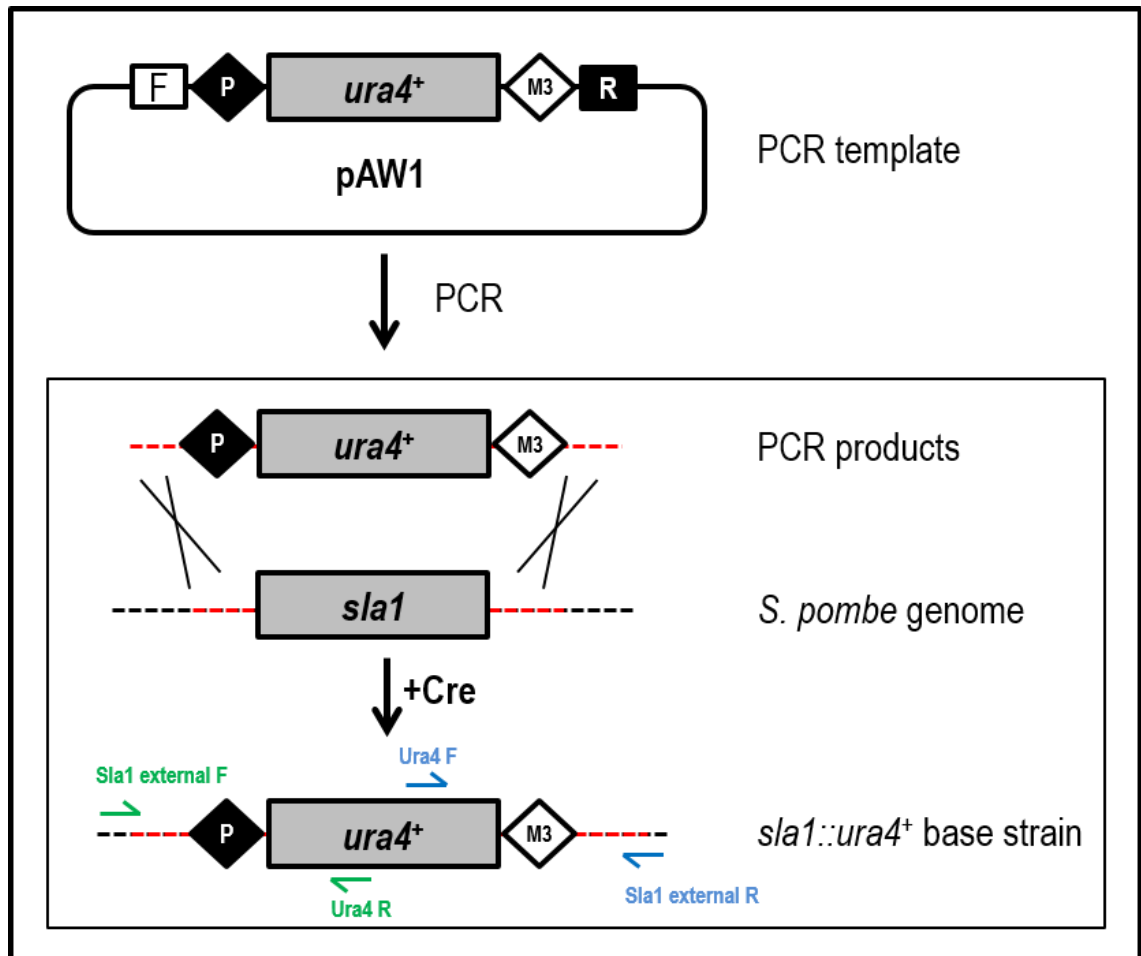


Figure 3.2 Construction of *sla1::ura4* base strain using standard homologous recombination techniques (Watson *et al*, 2008). Plasmid pAW1 was used as PCR templates—the loxP-*ura4⁺*-loxM3 cassette flanked by pFA6a derivative forward (F) and reverse (R) primer sites (Bahler *et al*, 1998), P = loxP sequence, M3 = loxM3 sequence. Sequences of pAW1-specific primers shown in Table 1 (Sla1 BS F and Sla1 BS R) contain the upstream and downstream sequences of the *sla1* gene, respectively, to generate fragments to be used to construct base strains for Cre-mediated cassette exchange. PCR product size is approximate 2.0 kb. Green and blue arrows are two sets of primers used for checking the replacement of the *sla1* gene by the loxP-*ura4⁺*-loxM3 cassette. This figure was adapted from Watson *et al*. (2008).

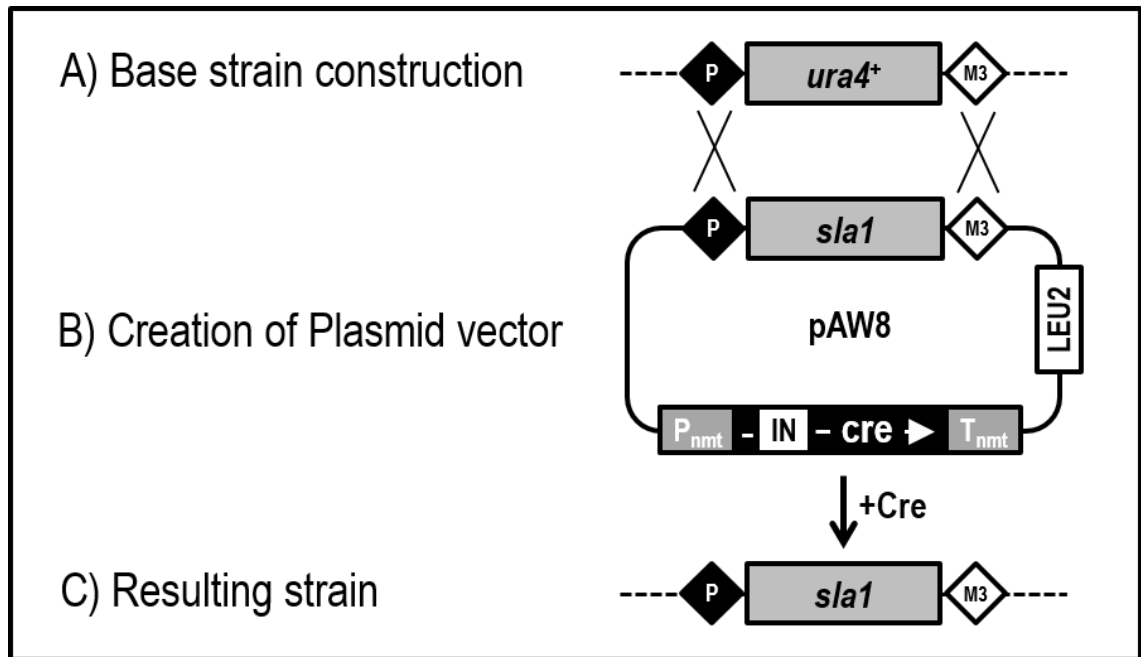


Figure 3.3 Schematic representation of Cre recombinase-mediated cassette exchange protocol. A) Construction of *sla1* base strain using standard homologous recombination techniques, B) introduction of Cre-expression plasmid containing *sla1* cassette sequences and C) expression of Cre recombinase resulting in *sla1* cassette exchange between the plasmid vector and chromosome, *sla1::ura4*. LEU2 = *S. cerevisiae* leucine selectable marker, *ura4*⁺ = *S. pombe* uracil selectable marker, P = loxP, M3 = loxM3, P_{nmt} = *S. pombe nmt41* promoter sequence, *S. pombe* T_{nmt} = *nmt* terminator sequence, cre = Cre recombinase coding sequence, IN = *S. pombe rad50* intron 1. This figure was adapted from Watson *et al.* (2008).

3.6 Phenotypic analysis of *sla1* mutants

The unSUMOylatable *sla1* mutants were used for phenotypic analysis and compared to wild type (shown in Figure 3.4). This figure includes a number of strains, namely (1) *S. pombe* wild type (*wt 501*, *b*-), (2) a *sla1* base strain in which *sla1* is replaced with *ura4* (*sla1::ura4*, *b*-), (3) a *sla1* wild type strain (*sla1-wt*, *b*-) obtained by transforming strain (2) with *wt-sla1*, (4) a *sla1* strain containing K106R (*sla1-K106R*, *b*-), (5) a *sla1* strain containing K263R (*sla1-K263R*, *b*-) (6) a *sla1* strain containing both K106R and K263R mutations (*sla1-RR*, *b*-), (7) a *sla1* base strain with *h*⁺ mating type (*sla1::ura4*, *b*⁺), (8) a strain containing *sla1* replaced with *Kan* (*sla1::kan*, *b*⁺, a Bioneer library strain). Strain 8 was crossed with *wt 501*, *b*- creating strain (9) with the opposite different mating type (*sla1::kan*, *b*-). Additionally, the *ura4* marker in the *sla1* base strain (2) was replaced by *NAT* in order to create (10) *sla1::NAT*, *b*- strain.

These *S. pombe* strains were exposed to a range of conditions to test for growth at 30°C and 37°C, sensitivity to rapamycin and/or ammonium chloride (NH₄Cl). The strains 1–6 were first analysed. Figure 3.4 shows that *sla1*Δ (*sla1::ura4*, *b*-) cells grew well in response to all the conditions, compared to wild type and *sla1-wt* strains. In addition, integration of neither of the single *sla1* mutations, K106R and K263R, nor the double mutation resulted in any alteration in phenotype. However this result was not consistent with recent studies by Cherkasova *et al.* (2012) who have shown that *sla1*-deleted *S. pombe* (*sla1::NAT*) grow slower than wild type cells on minimum medium.

Here, it was questioned whether there are other factors affecting the phenotype of the mutants, e.g. mating type or, replacement of *sla1* with different gene cassettes. This led to the construction of further strains (strains 7, 9–10) and the finding of a Bioneer library control strain (strain 8). These strains were grown under the same conditions as above. The result shows that different mating types of *sla1::ura4* cells (strains 2 and 7) do not affect the phenotype. In addition, our *sla1*Δ strains replaced by *ura4* and *NAT* cassette sequences did not have any effect on phenotype. In contrast, the *sla1::kan* cells (strains 8, *h*⁺ and 9, *h*-) had the altered phenotype. This suggests that gene cassettes may affect the phenotype of *sla1*Δ cells, but the mating type does not. The reason for this is not known.

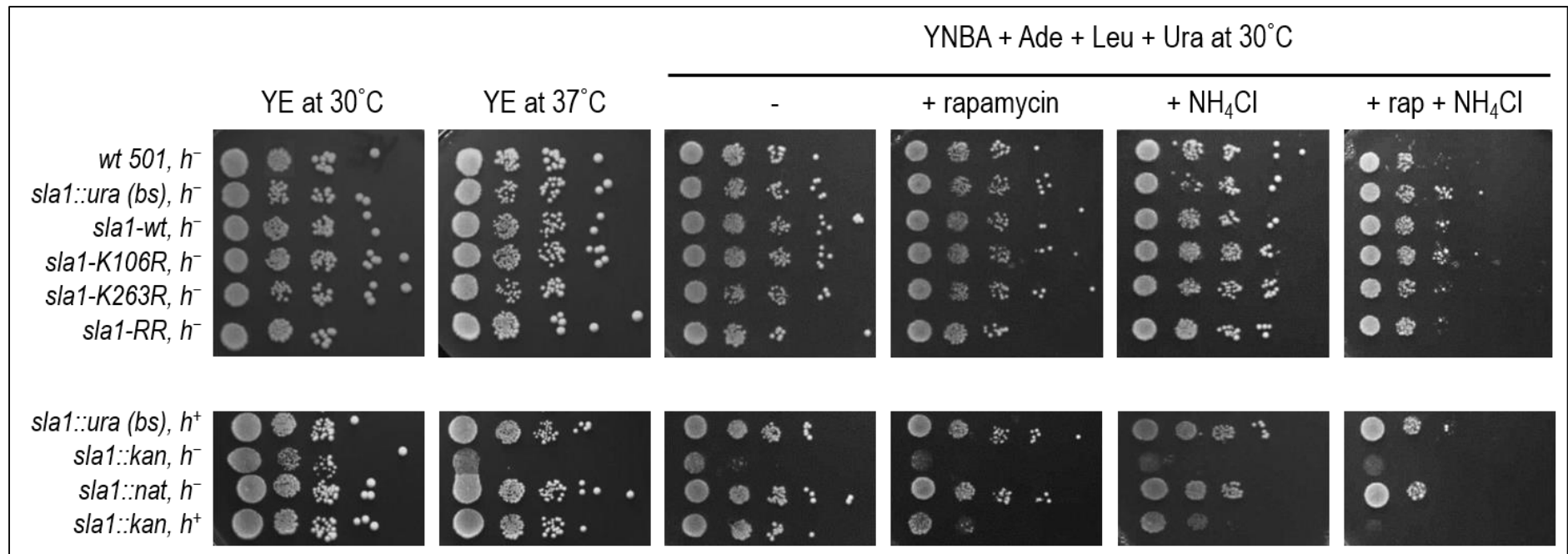


Figure 3.4 Analysis of the phenotype of unSUMOylatable *sla1* mutants. *S. pombe* wild type and various mutants were determined in response to growth temperature, rapamycin and ammonium chloride.

3.7 *S. pombe* eIF4G is SUMOylated *in vivo* but eIF3h is not

Since there was no detectable phenotype in unSUMOylatable Sla1 mutants, two other proteins were analysed. These were *S. pombe* eIF4G and eIF3h. Strains containing eIF4G and eIF3h tagged with the HA epitope at the C terminus were available for use (Dr. Felicity Watts). *S. pombe* cells containing HA-tagged eIF4G and eIF3h were separately transformed with pREP42-6xHis-SUMO (constructed by Dr. Lauren Small, University of Sussex). The pull-down experiments demonstrated that HA-tagged eIF4G was specifically recovered in the presence of His-tagged SUMO (lane 1 in Figure 3.5 A), but HA-tagged eIF3h did not pull down with His-tagged SUMO (lane 1 in Figure 3.5 B). This indicates that eIF4G is SUMOylated in *S. pombe*, but that eIF3h is not.

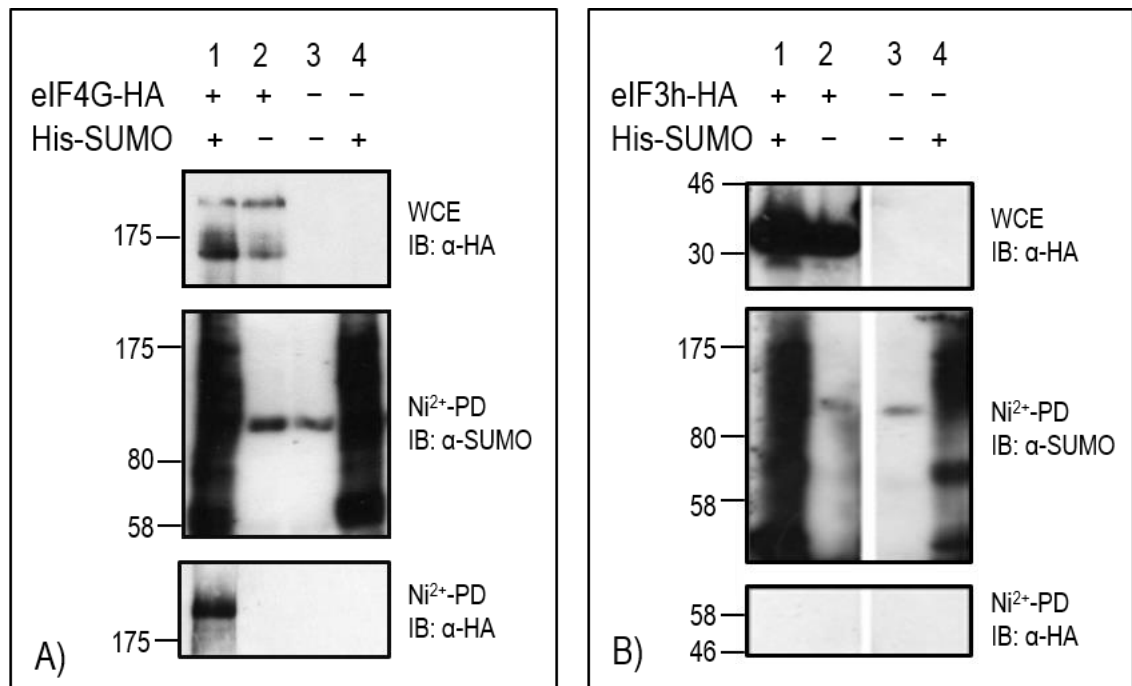


Figure 3.5 Western blotting of Ni²⁺ affinity purification for *S. pombe* eIF4G and eIF3h. His-tagged SUMO was affinity purified to test for SUMOylation of A) eIF4G and B) eIF3h. Whole cell extracts (WCE) and pulled down (PD) samples were analysed on 7.5 % gels. Blots were probed with anti-HA and anti-SUMO antisera.

3.8 The level of eIF4G modified by SUMO is increased after treatment with KCl

To determine whether SUMOylation of eIF4G was altered in response to different stress conditions, transformed HA-tagged eIF4G cells containing either pREP42-6xHis-SUMO or pREP42 6xHis empty vector were treated with either 100 µg/mL cycloheximide (Chx) or 1M KCl for 30 minutes and compared to untreated cells. Figure 3.6 shows that the level of SUMOylated eIF4G was increased 1.5 fold after treatment with 1M KCl, compared to the levels in untreated cells or cycloheximide-treated cells.

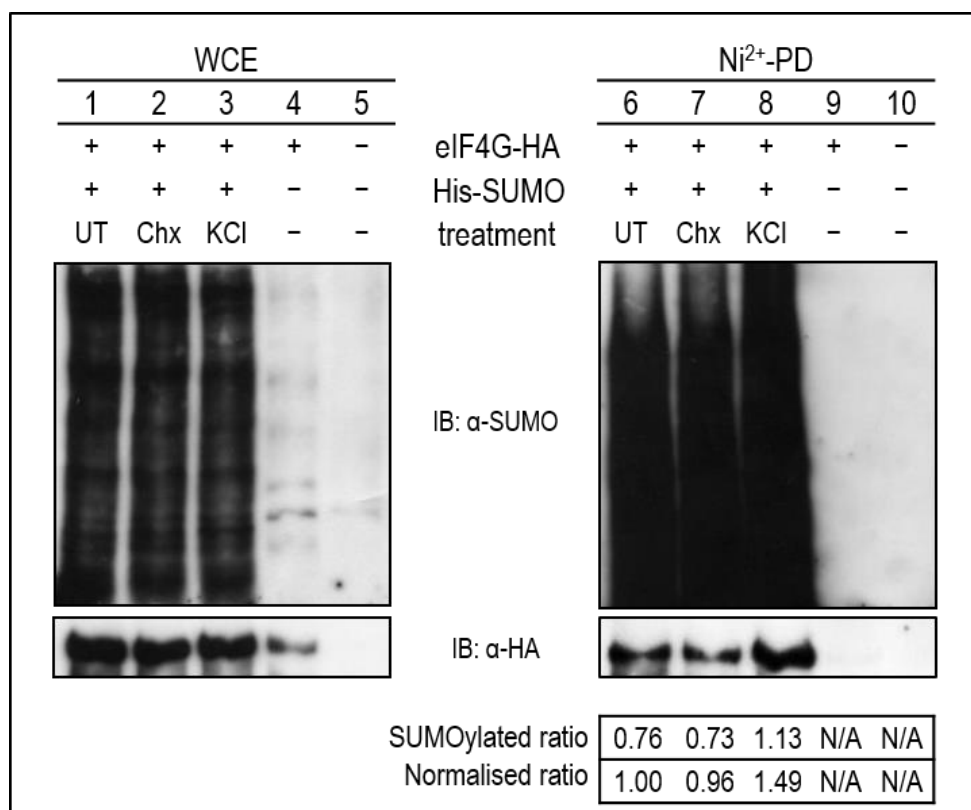


Figure 3.6 Western blotting of Ni²⁺ affinity purification for eIF4G in response to stresses. His-tagged SUMO was affinity purified to analyse SUMOylation levels of *S. pombe* eIF4G after the treatment with either 100 µg/mL cycloheximide (Chx) or 1M KCl for 30 minutes. Whole cell extracts (WCE) and pulled down (PD) samples were loaded on 7.5 % gels. Blots were probed with anti-HA and anti-SUMO antisera. Normalised ratio was calculated from the SUMOylated ratio of each stress condition (UT, Chx and KCl) divided by the SUMOylated ratio of non-treatment (UT). The SUMOylated ratio is the individual intensity of PD result (HA blot) which is divided by the intensity of its WCE result (HA blot).

3.9 *S. pombe* eIF4G and eIF3h protein are not formed in polysomes

Following the identification of eIF4G and eIF3h in complexes with Ulp2, gel filtration studies were used to confirm that eIF4G and eIF3h are indeed present in Ulp2-containing complexes (Dr. Felicity Watts) (as shown in Figure 2 in Jongjitwimol *et al*, 2014). To begin to identify the nature of these complexes, experiments were undertaken to determine whether the complexes were polysomes. Polysomes were prepared as described in 2.1.10 and analysis by western blotting. Figure 3.7 shows that Ulp2, eIF4G and eIF3h were present in the high molecular weight complexes containing polysomes in untreated cells. In cells treated with EDTA (which results in the disruption of polysomes), Ulp2 and eIF4G sediment in high molecular weight fractions. This indicates that the eIF4G and Ulp2 containing complexes are not polysomes. The identity of this complex, however, not known, but may be either stress granules or P-bodies.

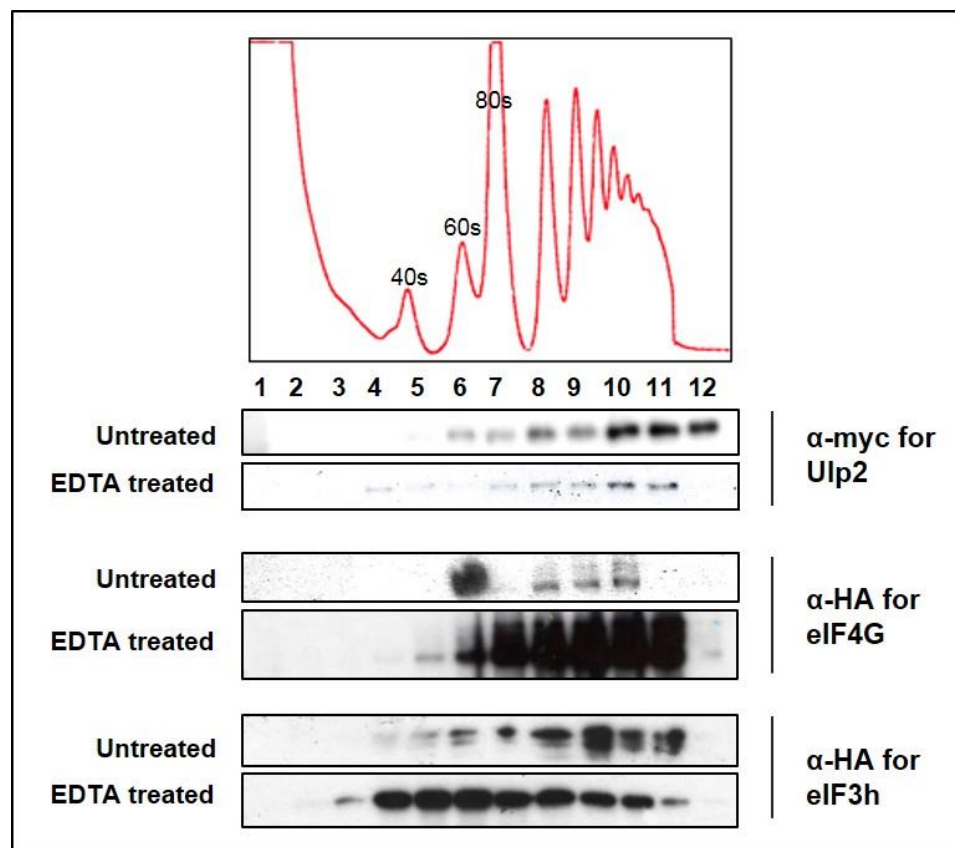


Figure 3.7 Western blotting of whole cell extracts from *Ulp2-myc + eIF4G-HA* and *Ulp2-myc + eIF3h-HA* strains. These cells were analysed from polysome fractions after treatment of EDTA compared to untreated condition. The fractions were loaded on 10 % gels gel. Blots were probed with anti-HA and anti-myc antisera.

3.10 Discussion

In this chapter, I analysed SUMOylation of some RNA-binding proteins in *S. pombe*, namely the *S. pombe* La homolog protein (Sla1), eIF4G and eIF3h. These three proteins are only some of several proteins that were co-purified with SUMO specific protease Ulp2. Other proteins (Table 1.4) may need to be further investigated for ability to be SUMOylated. It is interesting that these proteins are involved in processes such as RNA synthesis or processing, ribosome biogenesis and translation. This is consistent with recent studies indicating that a number of proteins required for ribosome biogenesis and RNA processing are SUMOylated (Panse *et al.*, 2006; Haindl *et al.*, 2008; Finkbeiner *et al.*, 2011). For example, SUMO-specific protease SENP3 has been demonstrated to associate with nucleophosmin and to be required for rRNA processing in ribosomal biogenesis (Haindl *et al.*, 2008; Finkbeiner *et al.*, 2011).

To begin with Sla1, it was demonstrated that this protein is modified by SUMO *in vivo*. This is consistent with the fact that the *S. pombe* La protein homolog interacts with SUMO protease Ulp2. To investigate SUMOylation of Sla1 expressed at endogenous levels, I undertook several attempts to C-terminally tag the *S. pombe sla1* gene with *myc* and HA epitopes as well as GFP sequences since antibodies against Sla1 were not available. However the gene tagging experiments were unsuccessful, suggesting that addition of a C-terminal tag to Sla1 is lethal in *S. pombe*. This was unexpected as deletion of the *sla1* gene was not lethal. In order to identify the SUMOylation sites of Sla1, our lab members have undertaken an *in vitro* SUMOylation assay and mass spectrometry. Sla1 has previously been demonstrated to be modified by SUMO in an *in vitro* assay (P. Taylor, Watts lab). Mass spectrometric analysis indicated that Sla1 is SUMOylated on lysine 106 and 263 (Dr. Brenda Mercer, University of Sussex).

To further phenotypically analyse unSUMOylatable mutants, *sla1* base strains (*sla1::ura4*) were created using the recombinase-mediated cassette exchange (RMCE) system for non-essential genes (Watson *et al.*, 2008) since Sla1 is a non-essential gene (Van Horn *et al.*, 1997). Using another RMCE system, the wild-type and three mutated pAW8-*sla1* plasmids were then transformed into the *sla1::ura4* base strain to achieve the *sla1-wt* strain, two strains containing a single lysine mutated to arginine (*sla1-K106R* and *sla1-K263R*) and one containing a double mutation (*sla1-RR*).

All unSUMOylatable *sla1* mutants were used for phenotypic analysis and compared to wild type strains and *sla1*-deleted strains (Figure 3.4). The *sla1* mutants did not have any affect to cell growth in YE (enriched) medium at 30°C. This is consistent with the fact that Sla1 is a non-essential gene (Van Horn *et al.*, 1997).

In response to other conditions (Figure 3.4), all *sla1* mutants had also no effect on cell growth in the enriched medium, except the *kan*-replacement *sla1*-deleted strains both h⁻ and h⁺ mating type (*sla1::kan*, h⁻ and h⁺). For example, the *sla1::kan* strains with h⁻ mating type (*sla1::kan*, h⁻) is sensitive for growth on YE medium at 37°C and on minimum medium, but the *sla1::kan* h⁺ strains is not. Both *sla1::kan* strains are defective in response to rapamycin and/or NH₄Cl, while others, in particular the *sla1Δ* strains containing *ura4* and *NAT* cassette sequences, are not. This suggests that gene cassettes may affect the phenotype of *sla1Δ* cells, but the mating type does not. The reason for this is not known, but similar discrepancies have been observed by other members of the GDSC with other genes. To conclude, investigating the role of SUMOylation of Sla1 was not possible using these mutant strains.

Next for the analysis of the SUMOylation of other proteins, the role of SUMOylation of eIFs has not been well studied even though a number of eIFs have been identified in proteomic screens as being SUMO targets (Table 1.5). Here, possible SUMOylation of two of the *S. pombe* eIFs that co-purified with TAP-Ulp2 were studied (Table 1.4), specifically, eIF4G and eIF3h. Both proteins are known to be present in high molecular weight complexes: eIF4G is part of the eIF4F complex and eIF3h is part of the eIF3 complex (Jackson *et al.*, 2010). It is here demonstrated that *S. pombe* eIF4G, but not eIF3h, is SUMOylated *in vivo* (Figure 3.5). This result is supported by recent studies where human eIF4G has been identified in proteomic screens as being SUMOylated (Matafora *et al.*, 2009; Bruderer *et al.*, 2011), while to date eIF3h has not been identified in these studies.

There were two obstacles that prevented further investigation of the role of SUMOylation of *S. pombe* eIF4G via the identification of the SUMOylation sites of the *S. pombe* eIF4G using an *in vitro* SUMOylation assay. The first difficulty occurred at the step of cloning the full length *S. pombe* eIF4G cDNA. This would be needed for *in vitro* SUMOylation assays in order to identify SUMOylated lysine residues using mass spectrometry. It has been previously shown that plasmids containing sequences encoding the N-terminal region of *S. pombe* eIF4G are unable to be tolerated in *E. coli*

(Hashemzadeh-Bonehi *et al.*, 2003). The reason for this is unknown, but is possibly due to the presence of a highly repeated sequence within the eIF4G coding sequence. Another factor was this highly repeated sequence in *S. pombe* eIF4G (but which is not found in the *S. cerevisiae* or human proteins). In the *S. pombe* protein, the highly repeated sequence contains 16 repeats of a perfect SUMOylation site consensus motif (AKRE), which would be likely to make it difficult to identify the SUMOylation sites, even if the full length eIF4G was able to be expressed in *E. coli*. Because of this, subsequent experiments were extended to human eIF4G in order to investigate the role of SUMOylation of this translation factors (Chapter 5).

Here, SUMOylation of *S. pombe* eIF4G was analysed in response to osmotic stress (1M KCl) that induces stress granules in *S. pombe*. The result (Figure 3.6) indicated that the level of SUMOylated eIF4G was increased after treatment with osmotic stress conditions compared to the levels in untreated cells or cycloheximide (Chx)-treated cells. The role of this modification is not known. However, there are two possibilities. The first, is that SUMOylation may be targeting eIF4G for degradation, possibly via the action of a SUMO-targeted ubiquitin ligase (STUbL). The second possibility is that SUMOylation may be targeting eIF4G to stress granules or P-bodies. Further studies conducted (Figure 3.7) here indicated that the eIF4G and Ulp2-containing complexes are unlikely to be polysomes. This would be consistent with SUMOylation targeting eIF4G to stress granules or P-bodies.

Chapter 4

Characterization of SUMOylation in Human Cell Lines

4.1 Introduction

In vivo studies indicated that *S. pombe* eIF4G is SUMOylated (Chapter 3). However, since it was not possible to clone full length *S. pombe* eIF4G which is needed for *in vitro* SUMOylation assays to identify modified sites, or for introduction of mutant alleles into the genome, attention was directed at mammalian translation initiation factors. Before analysis of these factors was analysed, the behaviour of a number of mammalian cell lines was analysed. Specifically, protein synthesis and SUMOylation were compared in a range of cell lines under different stress conditions, in order to determine whether there are any differences in different cell lines.

The cell lines chosen were HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells. MRC5 cells are normal fibroblast cells, being used to represent the normal behaviour of human cells. HeLa, PC3, MCF7 and MDA-MB231 cells were chosen as they are cancer cell lines three of which are believed to have altered SUMOylation levels. HeLa cells are one of most common cell lines used for studying the role of SUMOylation in many aspects *in vivo* e.g Hattersley *et al.* (2011), Geoffroy *et al.* (2010), Agbor *et al.* (2011). PC3 cells are one of a number of human prostate cancer cell lines. Studies have demonstrated that, in prostate cancer cells, SENP1 a SUMO-specific protease is overexpressed, compared to normal prostate cells (Cheng *et al.*, 2006b). In addition, recent studies have shown that the over-expression of Ubc9, a SUMO-conjugating enzyme, promotes tumour growth using MCF7 cells (Mo and Moschos, 2005; Mo *et al.*, 2005), and that this also enhances tumour invasion and metastasis using MDA-MB231 cells (Zhu *et al.*, 2010). These cells were therefore chosen in order to determine whether there are any alterations in SUMOylation levels or localisation in response to stresses.

4.2 Ionising radiation does not affect the alteration of global protein synthesis levels

As ionising radiation has been demonstrated to regulate protein synthesis (Lu *et al.*, 2006; Braunstein *et al.*, 2009). Global levels of protein synthesis were first compared in the different cell lines in response to ionising radiation (IR). Each cell line was grown to 60–80% confluence, and then treated with 3 Gy IR. Subsequently, untreated and IR-treated cells were incubated with ^{35}S -methionine in order to label newly synthesized protein and then allowed to recover for 60 and 120 minutes at 37°C (as previously described in section 2.5.20). Untreated and non-labelled cells were used as a baseline. Protein synthesis rate was shown as percentage of the rate of the untreated control at 60 minutes. It was calculated using values in cpm/ μg protein of each sample, minus the baseline value of untreated non-labelled samples. This becomes the adjusted value. Each of them was then divided by the adjusted value of the untreated labelled control at 60 minutes, then multiplied by 100. Figure 4.1 shows that, in untreated cells, protein synthesis approximately doubles after 120 minutes, compared to levels at 60 minutes for most cell types. Additionally exposure to IR has little effect on protein synthesis, except for MCF7 cells where the level is reduced, compare to that in untreated cells.

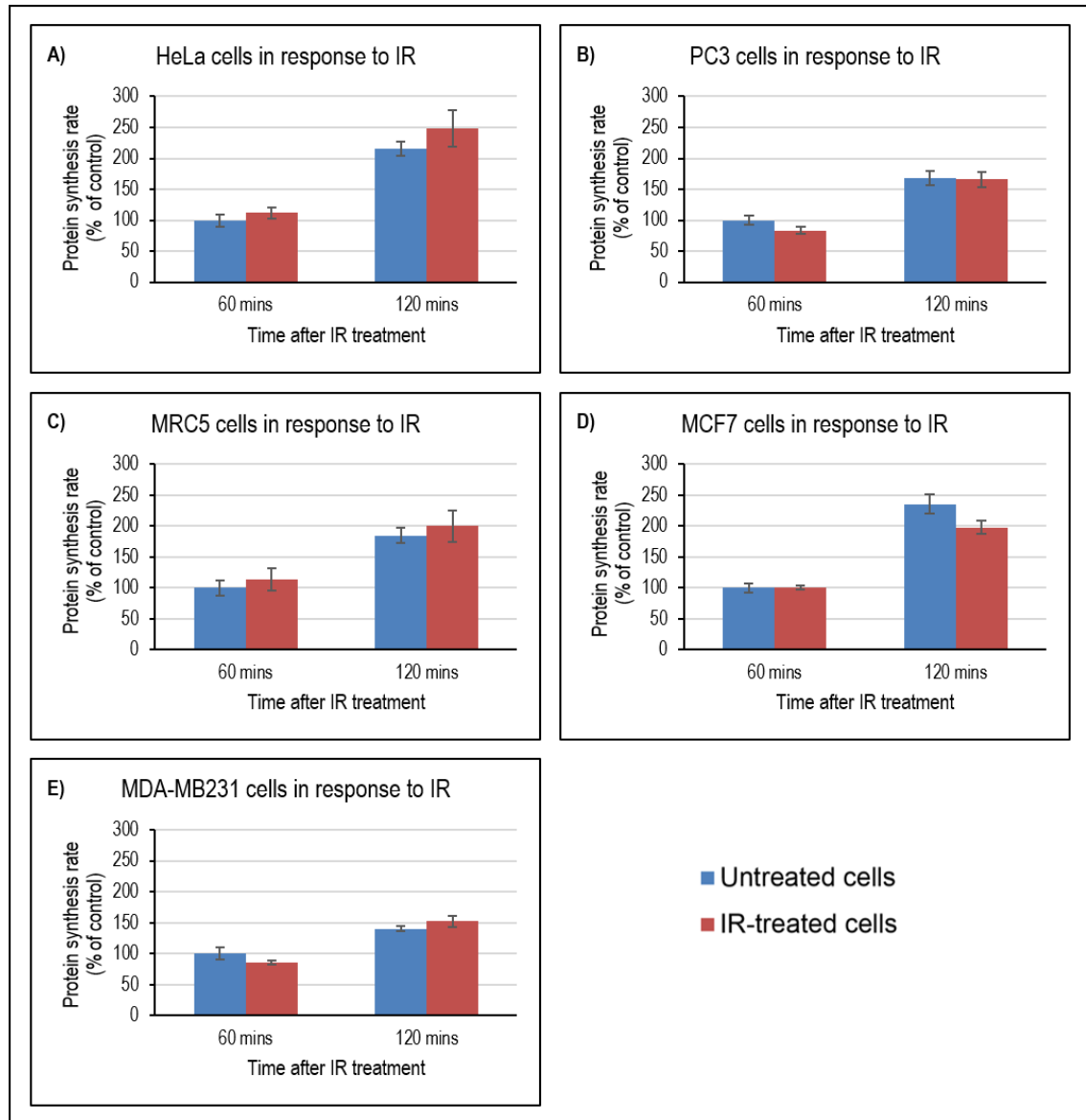


Figure 4.1 Protein synthesis assay using ^{35}S -methionine labelling. Different cell lines were treated and untreated with 3 Gy IR, and then allowed to reincubate for 60 and 120 minutes. A) HeLa cells B) PC3 cells C) MRC5 cells D) MCF7 cells and E) MDA-MB231 cells. Protein synthesis rate was shown as percentage, compared to the protein synthesis rate of the untreated control at 60 minutes after IR treatment. It was calculated using values in cpm/ μg protein of each sample, minus with the base line value of untreated non-labelled samples. Each adjusted value was then divided by the adjusted value of untreated-labelled cells after treatment 60 minutes, then multiplied by 100. The error bars are of two standard deviations.

4.3 Expression levels of SUMO1 and SUMO2/3 in different cell lines under stress conditions

Having analysed levels of protein synthesis in the different cell lines SUMOylation levels were then analysed in these cells. Specifically, levels of SUMO1 and SUMO2/3 conjugation were analysed in order to determine whether there were any differences in the cell lines under different stress conditions, namely sodium arsenite (AR) and ionising radiation (IR). Cells were grown to 60–80% confluence, and then treated and untreated with either 1 mM sodium arsenite or 3 Gy IR, followed by a reincubation for 30 minutes (as previously described in 2.4.6 and 2.4.7). Whole cell extracts from each cell line, treated and untreated with the different stresses, were analysed on 10% acrylamide gels, followed by western blotting with anti-SUMO1, anti-SUMO2/3 and anti-tubulin antibodies. Figure 4.2 panel A, indicates that using tubulin levels as controls, the levels of SUMO1 conjugates in MDA-MB231 are higher than in the other three cell lines. In HeLa cells and MRC5 cells, the levels of SUMO1 conjugates are slightly decreased in response to IR. AR and IR do not appear to alter the levels in MCF7 cells. Figure 4.2 panel B, indicates a difference between the cell types: specifically SUMO2/3 conjugates are distinctly different in MDA-MB231 compared to the other cell lines. In particular, low molecular weight SUMO2/3 species are present in high levels in MDA-MB231 cells. In MRC5 and MCF7 cells the pattern of SUMO2/3 conjugates is similar in untreated and IR-treated cells. In MDA-MB231 cells, the patterns of SUMO2/3 conjugates are also similar in untreated and IR-treated cells although the levels of high molecular weight species increase in response to IR. In response to AR, the pattern of SUMO2/3 conjugates is somewhat altered in all three cell lines; it is decreased in MCF7 and MDA-MB231 cells, but increased in MRC5 cells. In contrast, in HeLa cells, SUMO2/3 conjugates are decreased in response to IR. These results indicate that different stress conditions affect the levels of SUMOylation, particularly by SUMO2/3, in a range of human cell lines.

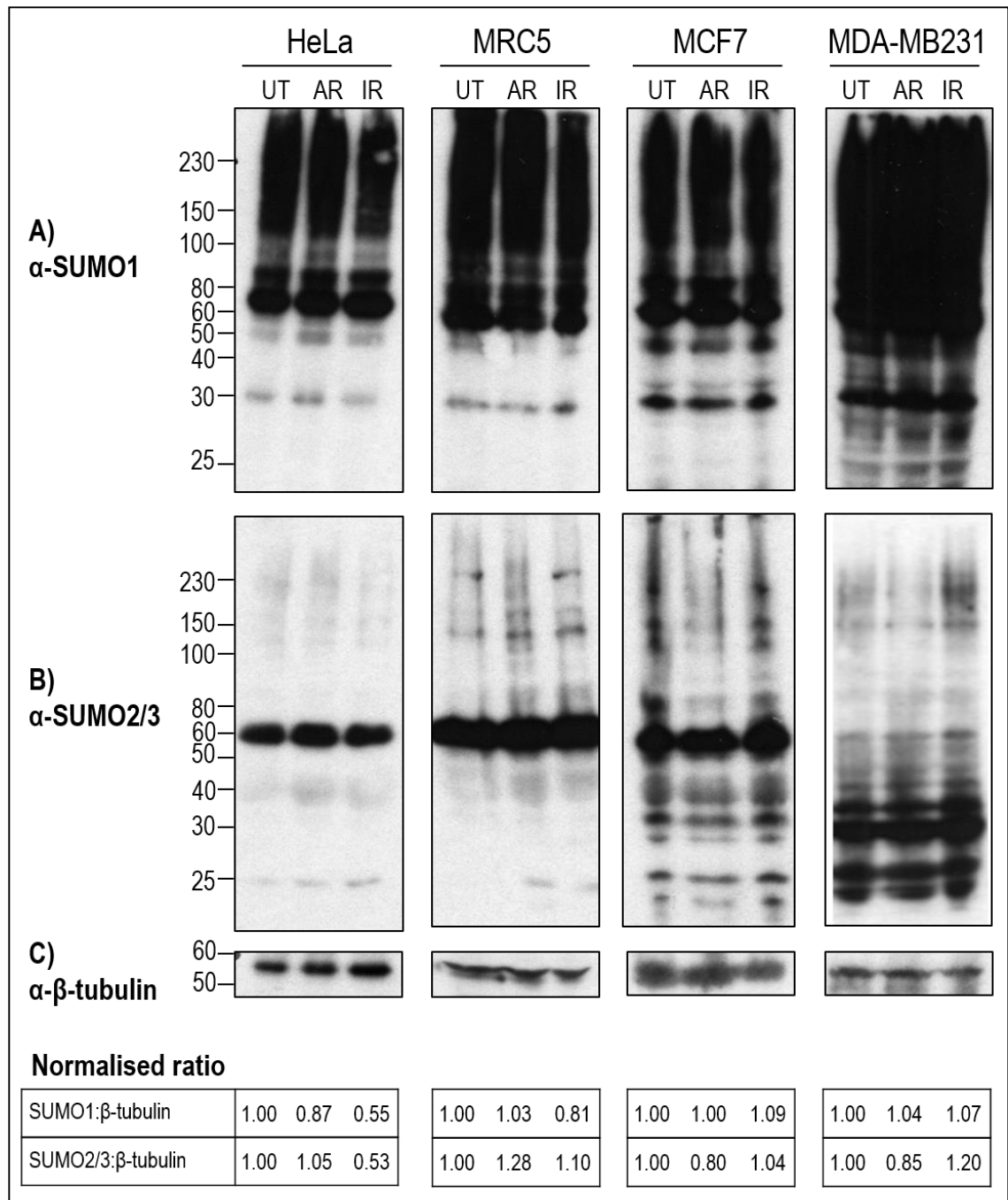


Figure 4.2 Western analysis of whole cell extracts from different human cell lines. Different cell lines were treated and untreated (UT) with either 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). Samples were analysed onto 10% SDS PAGE for western blotting with anti-SUMO1, anti-SUMO2/3 and anti- β -tubulin antibodies.

4.4 Localisation of SUMO1 in different cell lines after different ranges of treatments

Having analysed SUMO-conjugates, it was next of interest to analyse the localisation of SUMO in cells. HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells grown to 60–80% confluence were treated with and without either 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). Cells were reincubated at 37°C for 30 minutes. For microscopic analysis, untreated (UT) and treated cells (AR and IR) cells were incubated with mouse anti-SUMO1 antibody, followed by FITC anti-mouse IgG as described in section 2.4.8.

4.4.1 SUMO1 localisation in different cell lines (untreated condition)

Figure 4.3 shows that, in all untreated cell lines, SUMO1 (green) is present both in the nucleus and the cytoplasm, but is mainly present in the nucleus. HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells showed very similar patterns of localisation. However it is interesting that in MCF7 some of the SUMO1 localises at the nuclear periphery.

4.4.2 SUMO1 localisation in different cell lines in response to sodium arsenite (AR)

To study the localisation of SUMO1 in response to AR, all cell lines—HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells—were incubated with 1 mM sodium arsenite for 30 minutes (AR) before being stained with antibodies. Microscopic analysis (Figure 4.4) shows that SUMO1 (green) is still present in both the nucleus and the cytoplasm. However, in arsenite-treated cells, the intensity of SUMO1 staining in the nucleus is increased, compared to that in untreated cells. In addition, some of the SUMO1 formed foci in the nucleus of HeLa, PC3, MRC5 and MDA-MB231 cells. In PC3, MCF7 and MDA-MB231 cells, the SUMO1 foci localise at the periphery of the nucleus. It is possible that SUMO1 forms foci in the endoplasmic reticulum (ER) after AR treatment.

4.4.3 SUMO1 localisation in different cell lines in response to ionising radiation (IR)

HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells were treated with 3 Gy IR and then allowed to recover in an incubator for 30 minutes. Figure 4.5 shows that SUMO1 (green) is mainly present in the nucleus after IR treatment although there is a small

amount in the cytoplasm. All of the cell lines formed a number of SUMO1 foci in response to IR. These foci were more intense than those in the untreated and arsenite-treated cells. Unlike AR-treated cells, SUMO1 was not found to localise at the nuclear periphery in PC3, MCF7 and MDA-MB231 cells.

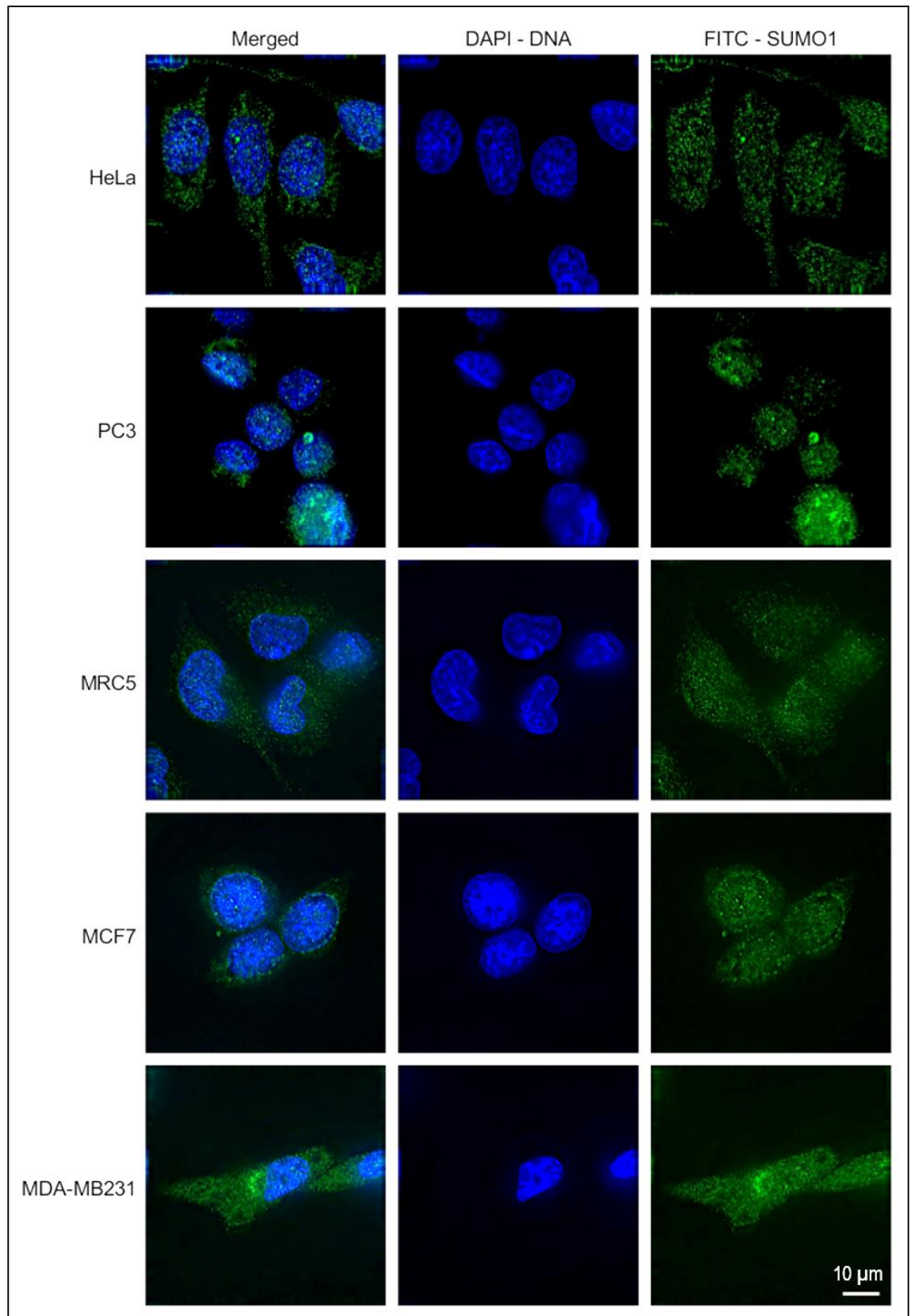


Figure 4.3 Immunofluorescence of SUMO1 localisation from untreated cells. HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells were determined the SUMO1 localisation without any treatment (UT).

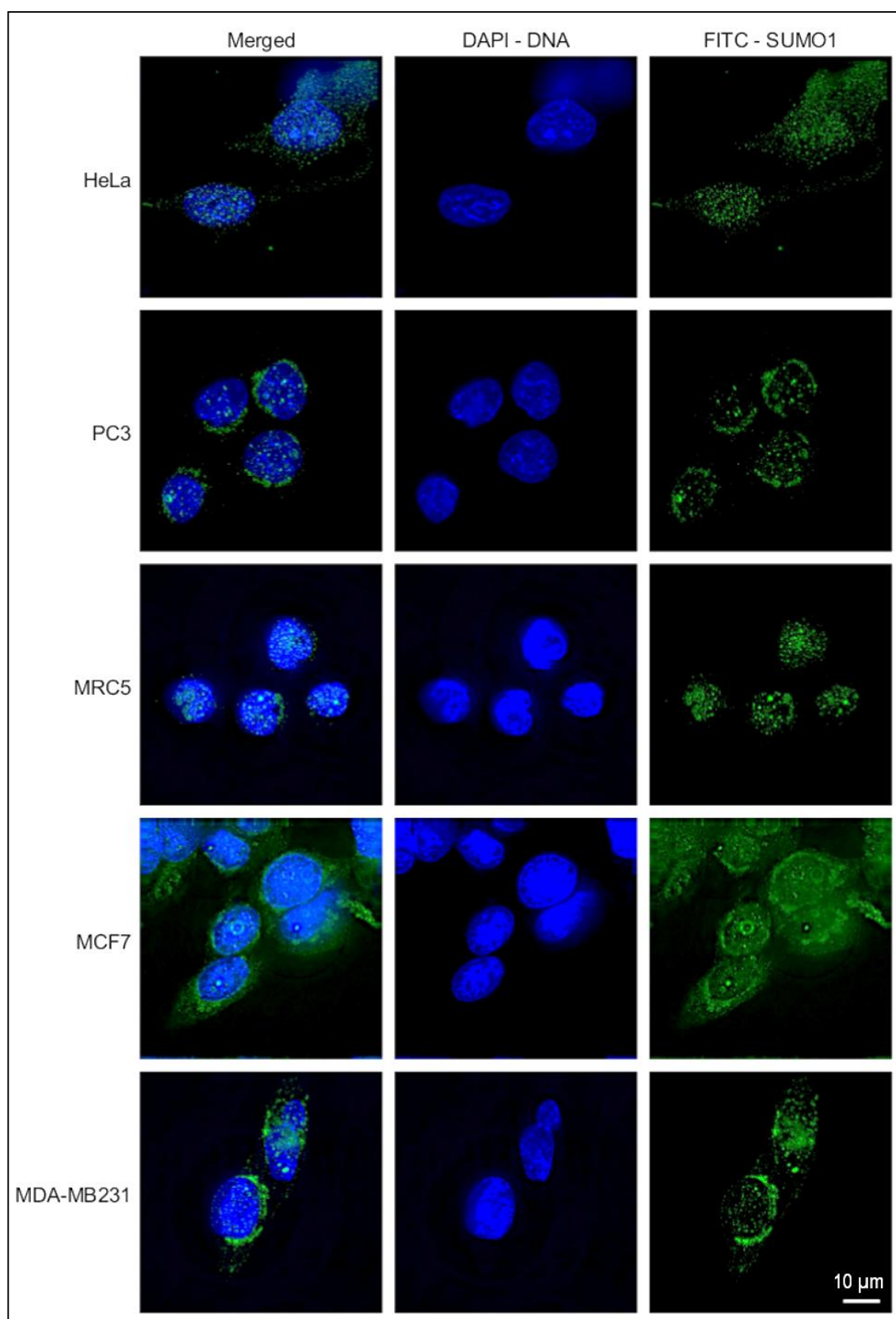


Figure 4.4 Immunofluorescence of SUMO1 localisation from AR-treated cells. The localisation of SUMO1 from HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells, treated with 1 mM sodium arsenite for 30 minutes, was analysed.

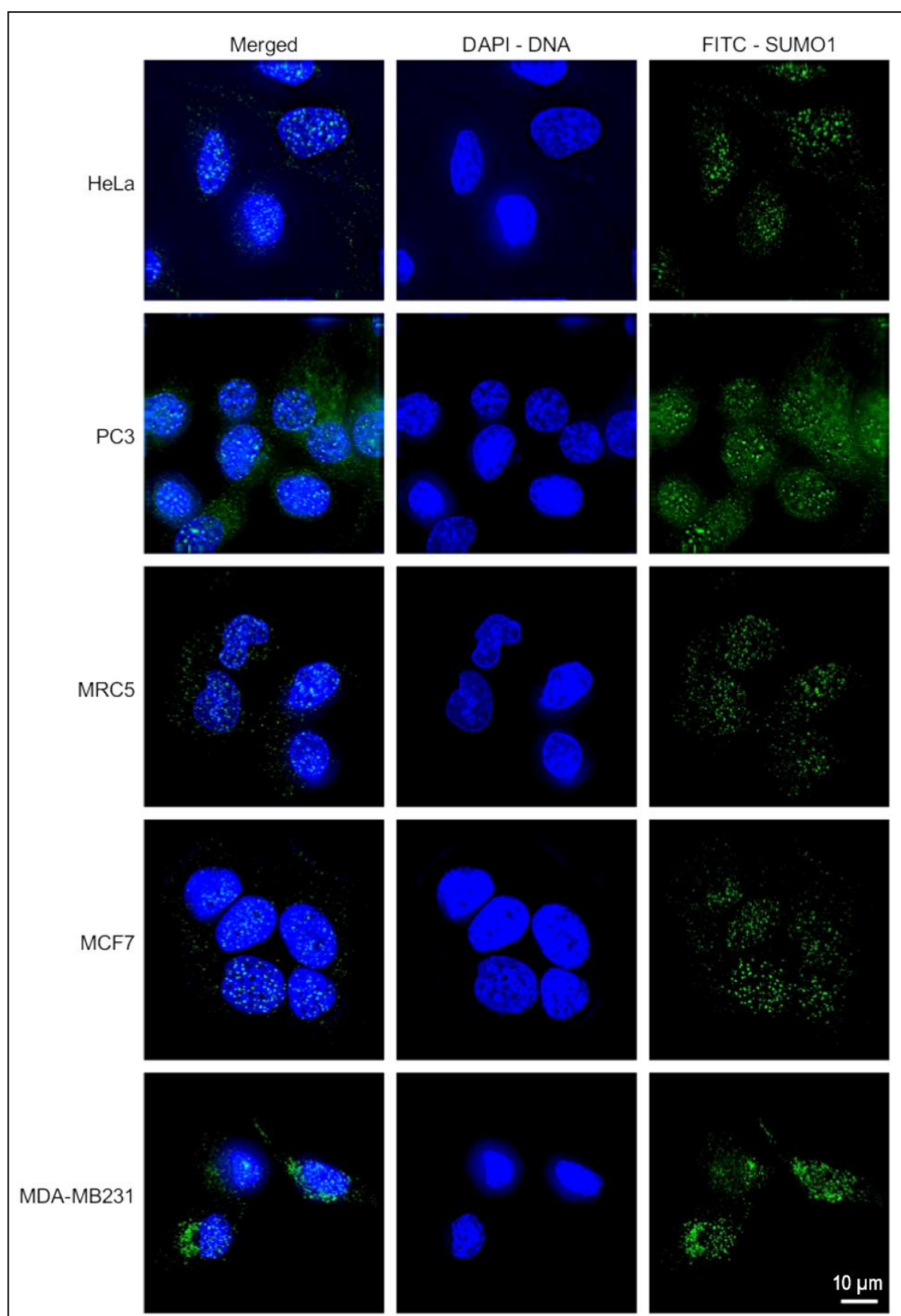


Figure 4.5 Immunofluorescence of SUMO1 localisation from IR-treated cells. The localisation of SUMO1 was analysed from HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells, treated with 3 Gy ionising radiation.

4.5 Discussion

The aim of this chapter was to analyse protein synthesis, levels of SUMOylation and SUMO localisation in a range of cell lines in response to stresses prior to further studies. Ionising radiation (IR) and sodium arsenite (AR) were chosen as stresses. IR has been demonstrated to regulate protein synthesis (Lu *et al.*, 2006; Braunstein *et al.*, 2009). Studies have demonstrated that protein synthesis is induced via the ATM and ERK pathway in IR-treated cells (Braunstein *et al.*, 2009). Sodium arsenite inhibits protein synthesis (Harding *et al.*, 2000; Souquere *et al.*, 2009). It results in the formation of stress granules which are sites where mRNA is stored before being used for reinitiation of protein synthesis or being degraded (Anderson and Kedersha, 2006).

Global levels of protein synthesis were primarily analysed in the different cell lines in response to ionising radiation (IR) using protein labelling assay. In all untreated cell types at 2 hours, the levels of protein synthesis approximately doubled after 2 hours relative to the levels at 1 hour. In most cell types, treatment with IR did not alter the levels of overall protein synthesis, i.e. levels again approximately doubles. Exceptionally, in IR-treated MCF7 cells reincubated for 2 hours, the rate of protein synthesis is lower than the control untreated MCF7 cells. This is consistent with some studies that have shown that translational arrest can occur in cells treated with IR. For example, it has been shown that IR induces the inhibition of the eIF4E/eIF4G interaction, affecting the cap-dependent translation pathway (Kumar *et al.*, 2000; Paglin *et al.*, 2005) or IR leads to reduced levels of eIF4G (Paglin *et al.*, 2005). To conclude, although no gross changes in the levels of protein synthesis after IR treatment, there may be changes in the translation of specific mRNAs that are undetectable in this assay.

SUMOylation levels in a range of human cell lines under different stress conditions were next analysed. In the different cell lines, there are small changes in the modification levels by SUMO1 and SUMO2 in response to AR and IR. In particular, in HeLa cells, the levels of SUMO1 and SUMO2/3 conjugates were decreased in response to IR. In MRC5 cells, there was a small decrease of SUMO1 conjugates in response to IR, but an increase of SUMO2/3 conjugates in response to arsenite. In contrast, in MCF7 and MDA-MB 231 cells, arsenite decreased the levels of SUMO2/3 conjugates while IR increased the levels of SUMO2/3 conjugates in MDA-MB231 cells. These results suggest that both AR and IR affect the levels of SUMOylation in a range of human cell lines.

The SUMO1 localisation was lastly investigated, in order to identify any differences in the cell lines following exposure to AR and IR. In all cell lines, untreated and treated with either AR or IR, SUMO1 mainly localises in the nucleus but it is also found in the cytoplasm. Without any treatment, HeLa, PC3, MRC5, MCF7 and MDA-MB231 cells have quite similar patterns of SUMO1 localisation, except MCF7 where SUMO1 sometimes localises at the nuclear periphery. In most AR-treated cells, except MCF7, SUMO1 is predominantly in the nucleus where it is found to form foci. In addition, in PC3, MCF7 and MDA-MD231 cells, SUMO1 foci are present at the periphery of the nucleus, suggesting that SUMO1 may form foci in the endoplasmic reticulum (ER) after AR treatment. There are a number of SUMO1 foci formed in the nucleus of cells exposed to IR which is more than in cells exposed to AR. This is consistent with the modification of PML by SUMO1 which forms foci (PML bodies) in the nucleus after cells are treated with arsenite (Müller *et al.*, 1998; Geoffroy *et al.*, 2010). This is known to result in an increase in the colocalisation of PML with a number of proteins in the PML nuclear bodies at the sites of DNA damage after IR e.g. TopBP1 (Xu *et al.*, 2003), SUMO1 (Dellaire and Bazett-Jones, 2004). A similar analysis of SUMO2/3 localisation was not undertaken at this time because our anti-SUMO2/3 antibodies cross reacted with other epitope(s) in the cells, thus confusing the images.

In conclusion, the levels of global protein synthesis are not grossly altered in a number of human cell lines after exposure to IR, apart from MCF7 cells where a small decrease in the rate of global protein synthesis was observed. SUMOylation levels were shown to vary, depending on the cell type and treatment. For the SUMO1 localisation, SUMO1 mainly localises in the nucleus, and can be found in foci when cells were treated with AR or IR.

Chapter 5

Analysis of SUMOylation of Human eIF4G

5.1 Introduction

Although it was possible to demonstrate that *S. pombe* eIF4G is SUMOylated. There are a number of factors, described in Chapter 3, that make the identification the SUMOylation sites of the *S. pombe* eIF4G difficult. Briefly, one was the inability to express the full length *S. pombe* eIF4G in *E. coli*, and another was the presence of a highly repeated sequence in the *S. pombe* protein. Attention was therefore redirected to the analysis of the SUMOylation of mammalian initiation factors, in particular human eIF4G and eIF4A.

This chapter focuses on the investigation of SUMOylation of human eIF4G, which is one of the proteins identified in proteomic screens as being a SUMOylated target (Table 1.5). To confirm that human eIF4G is SUMOylated, two approaches were taken, the first was to determine whether eIF4G is SUMOylated *in vitro*, which would allow identification of SUMOylation sites by mass spectrometry, and the second was to determine whether it is also SUMOylated *in vivo*.

5.2 Expression and purification of SUMO components for the *in vitro* SUMOylation assay

In vitro SUMO modification assays of protein targets have been developed as an effective tool for the analysis of SUMOylation and identification of SUMOylation sites (Johnson and Blobel, 1997; Desterro *et al.*, 1998; Okuma *et al.*, 1999; Ho *et al.*, 2001). In order to undertake the *in vitro* assay it was first necessary to purify the components of the SUMOylation machinery. The recombinant SUMO components expressed and purified in this study were the heterodimeric SUMO-activating E1 enzyme (6xHis-Rad31 and GST-Fub2), the SUMO conjugating E2 enzyme (GST-Hus5), the SUMO E3 ligase (6xHis-Pli1) and SUMO (6xHis-SUMO-Tr-GG).

5.2.1 Isolation of SUMO activating enzyme E1 (SAE; Rad31 and Fub2) co-expressed in *E. coli* system

The *S. pombe* SUMO-activating E1 enzyme (SAE) is a heterodimeric protein which consists of Rad31 (6xHis-tagged) and Fub2 (GST-tagged). To obtain the complex form of the E1 enzyme, pET28a containing Rad31 and pGEX containing Fub2 were co-transformed and proteins were expressed and purified from a one litre of BL21 (DE3) *E. coli* culture. The heterodimeric enzyme was isolated in a one-step purification using Ni²⁺ agarose beads (see section 2.5.13). 300 µl of elution buffer was used to elute the recombinant SAE from the agarose beads. The protein concentrations of the 1st–5th elution (E1–E5) fractions were measured using a Bradford assay. The protein concentrations of the E1–E5 fraction were 1.5, 1.5, 0.1, 0.0 and 0.0 mg/ml, respectively. To check the purity of the complex, 10 µl of each fraction was loaded onto a 12.5% acrylamide gel. The result in Figure 5.1 indicates the fractions E1, E2 and E3 have a good yield of the SAE complex with the recombinant proteins His-Rad31 and GST-Fub2, represented by approximately 96 kDa and 36 kDa bands, respectively. To store the sample for further use, 30 µl of 100% glycerol was added into the fractions E1–E3. The samples were then aliquoted and kept at –80°C.

5.2.2 Expression and purification of the SUMO conjugating enzyme E2 (Hus5)

The SUMO conjugating enzyme E2 (GST-tagged Hus5) was expressed and purified from a one litre culture. GST-tagged Hus5 was expressed from the pGEX plasmid. The GST fusion protein was purified as previously described (see section 2.5.14). 300 µl of each fraction were collected during elution. The protein concentrations of the fractions E1–E5 were 0.8, 1.8, 0.1, 0.05 and 0.0 mg/ml, respectively using Bradford assay. To check the purity and size of GST-tagged Hus5, 10 µl of each fraction was loaded onto a 12.5% acrylamide gel. It can be seen in Figure 5.2 that the 44 kDa recombinant Hus5 was purified with good purity in fractions E1, E2 and E3. These samples were mixed with 30 µl of 100% glycerol. The samples were then aliquoted and frozen at –80°C.

5.2.3 Expression and purification of the SUMO ligase E3 (6xHis-tagged Pli1)

6xHis-tagged Pli1 was expressed from the pET15b plasmid in a one litre culture and then isolated by Ni^{2+} purification as previously described (see section 2.5.13). 300 μl of each elution fraction were collected and the protein concentration was determined using a Bradford assay. The purification of the recombinant Pli1 was attempted several times but due to the instability of the protein it was difficult to get a high yield with good purity (data not shown). Thus the SUMO ligase E3 protein (His-Pli1) used here was a kind gift from Dr. Brenda Mercer (University of Sussex).

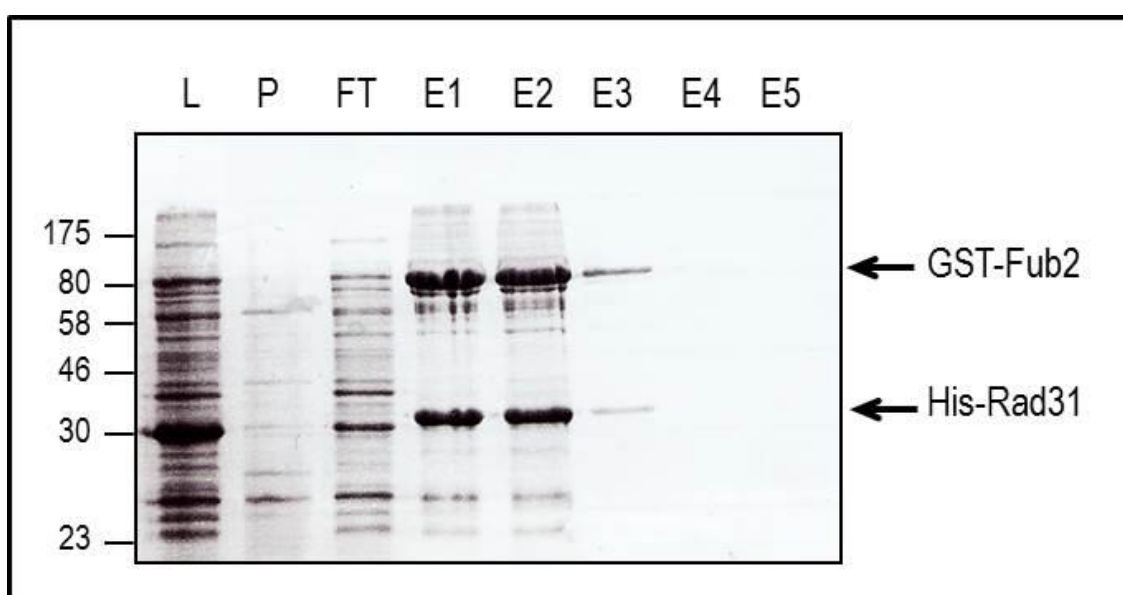


Figure 5.1 Coomassie blue staining for the purification of SAE E1 complex. 10 μl of each elution fraction was loaded onto a 12.5% acrylamide gel (L = lysate, P = cell pellet, FT = the first flow through, E1-E5 = the elution fractions 1st–5th). From one litre of culture, the protein concentrations of the E1–E5 fractions were purified at 1.5, 1.5, 0.1, 0.0 and 0.0 mg/ml, respectively. The higher band (~ 96 kDa) represents GST-tagged Fub2 of which the molecular weight of Fub2 and GST are 70.6 and ~ 26 kDa, respectively. The lower band (~ 36 kDa) represents His-tagged Rad31 of which the size of Rad31 is 34.7 kDa.

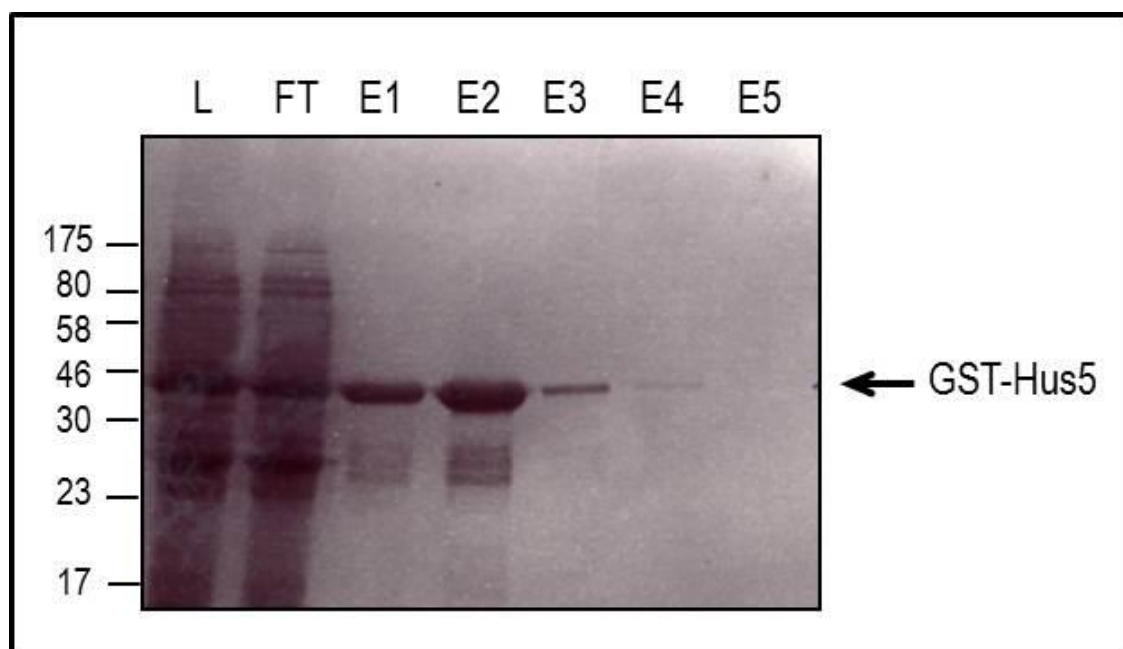


Figure 5.2 Coomassie blue staining for GST-tagged Hus5 purified. To observe purity and size of the protein purified, 10 μ l of each elution fraction (300 μ l in total volume) was loaded onto a 12.5% acrylamide gel. (L= lysate, FT= the first flow through, E1-E5= the eluted fraction 1st–5th). The protein concentrations of the E1–E5 fractions were 0.8, 2.0, 0.1, 0.05 and 0.0 mg/ml, respectively. The ~44 kDa band represents Hus5 (17.9 kDa) tagged with GST (~26 kDa).

5.2.4 Expression and purification of SUMO-Tr-GG

As one of the aims of using the SUMOylation assay was to identify SUMOylation sites, it was necessary to use a modified form of SUMO. Mass spectrometry is an important tool for the identification of the specific molecular mass of peptides representing as mass-to-charge ratio (m/z). It can be used to detect sites of post-translational modification. Most proteases recognize a particular amino acid when cleaving polypeptide chains. For example, trypsin (commonly used in this study) mainly cleaves C-terminal to lysine (K) and arginine (R), except when either is followed by proline (P). When protein targets modified by *S. pombe* wild type SUMO are digested by trypsin, the large mass peptide (IRPDQTPAELDMEDGDQIEAVLEQLGG) will remain covalently attached to the lysine of the target's peptide. This may be difficult to detect by mass spectrometry as it is quite large already and will be increased in size by addition of target sequences. SUMO-Tr-GG is a truncated form of SUMO with a diglycine motif (SUMO-GG) at the C-terminal region and a trypsin cleavage site adjacent to the motif. If protein targets modified by SUMO-Tr-GG version are digested by

trypsin, a smaller mass diglycine (GG) motif instead remains covalently attached to the lysine of target's peptides. This improves the detection by mass spectrometry facilitating identification of lysine residues modified by SUMO.

The SUMO-GG sequence (encoding the mature form of SUMO) in pET15b was mutated at amino acid position 109 from leucine (L) to arginine (R), creating SUMO-GG L109R or SUMO-Tr-GG, which is a SUMO-GG form with a trypsin cleavage site adjacent to the diglycine motif (gift from Dr. Lauren Small, University of Sussex). The sequence alignment between SUMO and SUMO-Tr-GG is shown in Figure 5.3.

SUMO-Tr-GG tagged with 6xHis epitope was expressed in a one litre culture of BL21 *E. coli*. Ni²⁺-purification was carried out as previously described (see section 2.5.13). 300 µl of each elution fraction was collected. The protein concentration of 1st–5th elution (E1–E5) fraction measured using a Bradford assay were 3.5, 8.0, 2.9, 0.1 and 0.0 mg/ml, respectively. To check the purity of the protein, 10 µl of each fraction was loaded onto the 12.5% acrylamide gel. The gel was stained with Coomassie blue, shown in Figure 5.4. The result shows that the purification of His-SUMO-Tr-GG was good with good yields. Samples were preserved in glycerol (10%). Aliquots were frozen at –80°C.

SUMO WT	MSESPSANISDADKSAITPTTGDTSQQDVKPSTEHINLKV	40
SUMO-Tr-GG	MSESPSANISDADKSAITPTTGDTSQQDVKPSTEHINLKV	40

SUMO WT	VGQDNNEVFFKIKKTTEFSKLMKIYCARQGKSMNSLRFLV	80
SUMO-Tr-GG	VGQDNNEVFFKIKKTTEFSKLMKIYCARQGKSMNSLRFLV	80

SUMO WT	DGERIRPDQTPAELDMEDGDQIEAVLEQLGGCTHLCL	117
SUMO-Tr-GG	DGERIRPDQTPAELDMEDGDQIEAVLEQGG-----	111
	***** **	

Figure 5.3 The alignment between the sequences of *S. pombe* wild type SUMO and SUMO-Tr-GG (SUMO-GG L109R). The blue triangles show the position of trypsin cleavage sites where the larger sequences (IRPDQTPAELDMEDGDQIEAVLEQLGG) of SUMO WT and the smaller diglycine sequences (GG) of SUMO-Tr-GG remain covalently attached to the modified lysines of target peptides after digestion of the protein targets by trypsin.

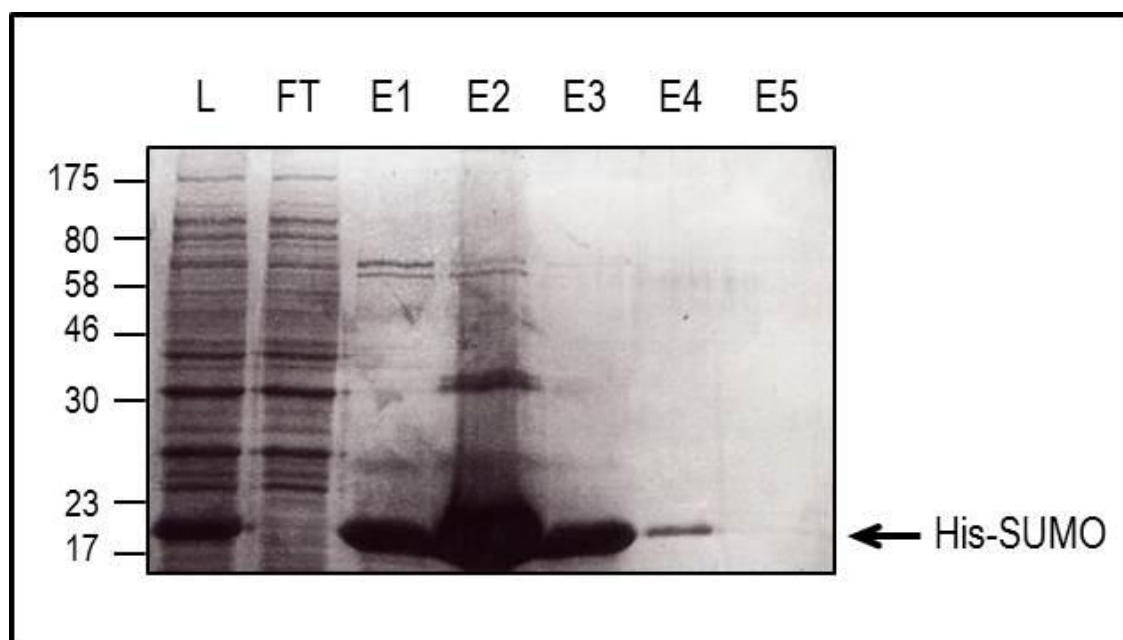


Figure 5.4 Coomassie blue staining indicating purification of His-tagged SUMO-Tr-GG purified. A 12.5% gel was stained with Coomassie blue (L= lysate, FT= the first flow through, E1–E5= the elution 1st–5th). The protein concentrations of E1–E5 fractions were 3.5, 8.0, 2.9, 0.1 and 0.0 mg/ml, respectively.

5.2.5 Evaluation of the enzymatic activity of all recombinant SUMO components in *in vitro* SUMOylation assay

To evaluate the enzymatic activity of the SUMOylation components, all purified proteins were analysed for ability to form SUMO chains using the conditions described Ho *et al.* (2001). The reaction was incubated at 30°C for 2 hours. Previously prepared SUMOylation components (from Dr. Brenda Mercer and Dr. Lauren Small, University of Sussex) were used as a set of positive (lane 1–4 in Figure 5.5) and negative controls (lane 5). It can be seen that the previously prepared SUMOylation components in lanes 3 and 4 (containing twice as much SAE E1 as used in lanes 1 and 2) are capable of SUMO chain formation as evidenced by the high molecular weight species. The reactions in lanes 6–11 used the SUMOylation components purified from this study (except Pl1, made by Dr. Brenda Mercer).

The 1st elution of SAE (lane 6 and 7) and the 2nd SAE fraction (lane 8 and 9) show results similar to those observed with the positive control in lanes 3 and 4. This shows that both elutions of SAE enzyme have a good ability to form SUMO chains in an *in vitro* SUMOylation assay. In contrast, the third elution fraction (lane 10 and 11) has very little activity, with little SUMO chain formation, being similar to the negative control in lane

5 which is in the absence of SAE. This suggests that the first and second elution fractions of SAE have good enzymatic activity with or without Pli1.

The function of the other components (Hus5 and SUMO) was also assayed for ability to form SUMO chains (data not shown). Both proteins were shown to be functional.

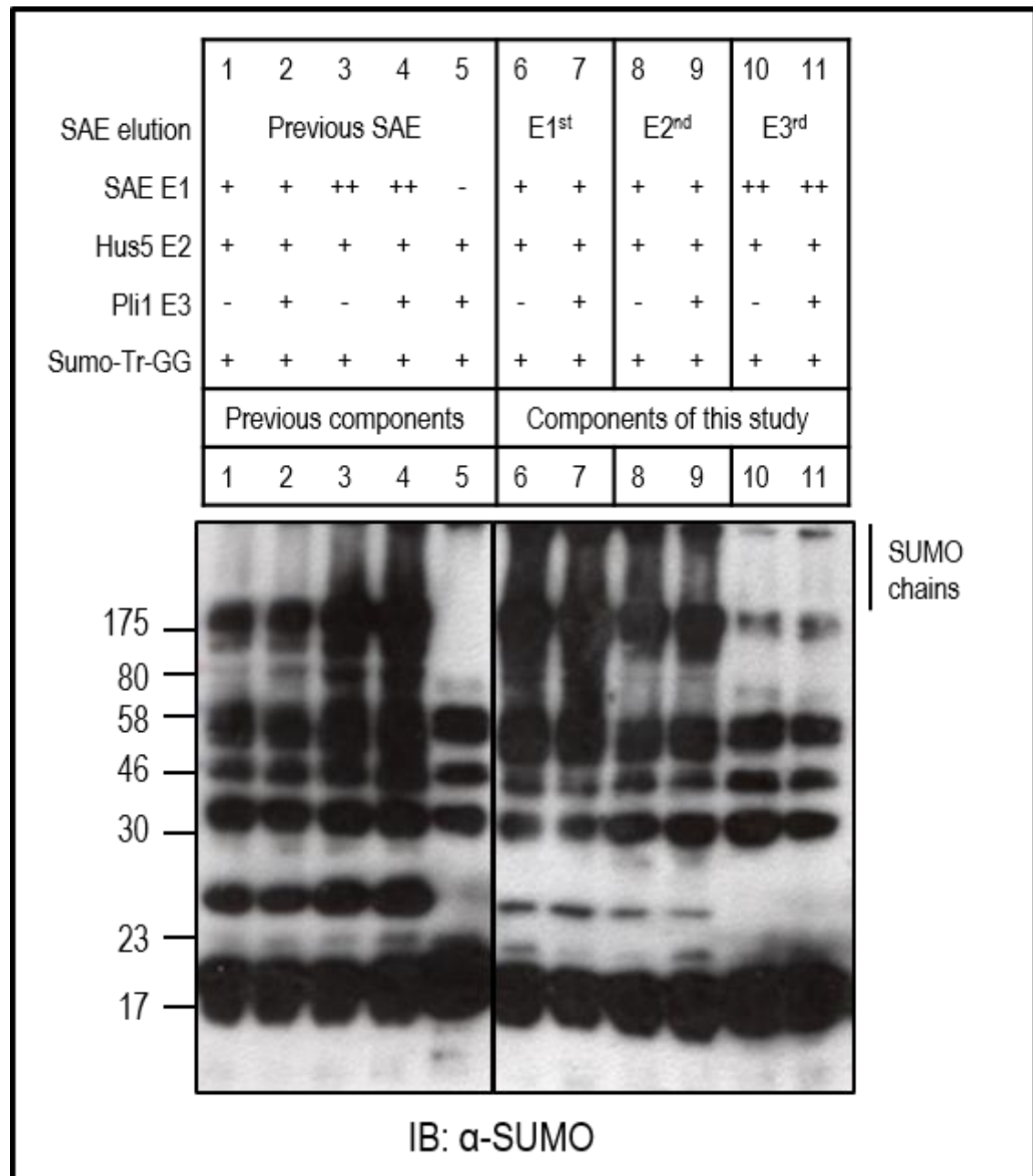


Figure 5.5 Assaying the activity of the purified SUMOylation components. Western blotting of *in vitro* SUMOylation assay with anti-SUMO antisera. Lanes 1–5 are the previously purified components made by Dr. Mercer and Dr. Small (Sussex), while lanes 6–11 are the proteins purified in this study.

Table 5.1 The amount of SUMOylation components used in this study compared to those used in the study of Ho *et al* (2001).

Component	Amount used by Ho <i>et al</i> (2001)	Amount used in the current study
SAE (E1)	0.5 µg	Elution 1 and 2: 0.6 µg Elution 3: 0.4
Hus5 (E2)	3.0 µg	Elution 1 and 2: 3.0 µg
Pli1 (E3)	NA (optional)	Dr. Mercer: 1 µg
SUMO-GG	10.0 µg	-
SUMO-Tr-GG	-	Elution 1–3: 8.0 µg

5.3 Human eIF4G expressed in *S9* insect cells is SUMOylated *in vitro*

5.3.1 Expression and purification of His-Flag tagged human full length eIF4G performed in *S9* insect cells

S9 insect cells were infected with Baculovirus encoding human 6xHis-3xFLAG tagged eIF4G1 (the recombinant Baculovirus was provided by Prof. Simon Morley, Sussex). After His-Flag tagged eIF4G was expressed in *S9* insect cells in a 400 ml suspension culture grown for 72 hours, cells were harvested for the isolation of the recombinant protein using Ni²⁺ agarose beads under native conditions as previously described in the section 2.5.16. In the elution step, 500 µl of each elution fraction was collected. Bradford assay was used to measure the protein concentration of each fraction (E1–E5) which was at 3.30, 1.66, 0.31, 0.03, 0.00 mg/ml, respectively. To check the purity of the recombinant protein, 10 µl of lysate (L), flow through (FT), each elution (E1–E5) and beads were loaded onto two 10% acrylamide gels, of which the first was stained with Coomassie blue and the second was for western blotting. Figure 5.6 demonstrates that the recombinant eIF4G was expressed and purified with high yield in the elution fractions E1–E3. The presence of lower molecular weight species is likely due to proteolysis of eIF4G.

5.3.2 Human eIF4G expressed in *S9* insect cells is SUMOylated in the presence of Pli1 *in vitro*

Purified human eIF4G was next assayed for ability to be SUMOylated *in vitro*. 6.6 or 13.2 µg of protein purified (from elution 1 = E1 and elution 2 = E2) was incubated with SUMOylation components as shown in Table 5.1. Products were analysed by SDS PAGE, followed by staining with Coomassie blue and western blotting with anti-eIF4G and anti-SUMO antisera (Figure 5.7). Coomassie blue staining and western blotting with anti-eIF4G antisera indicate that, in comparison to purified eIF4G (Figure 5.6), there has been degradation of the eIF4G protein. Nevertheless, Coomassie blue staining indicates high molecular weight species in lanes 6–8, where are not present in the negative control lanes 2–3. Western blotting with anti-eIF4G also indicates high molecular weight species in lanes 6, 7 and 9. Western blotting with anti-SUMO antisera also indicates high molecular weight SUMO-containing species, which are more abundant in the presence of Pli1 (lane 7 and 9) than in the absence of Pli1 (lane 6 and 8). These results suggest that human eIF4G is SUMOylated *in vitro*.

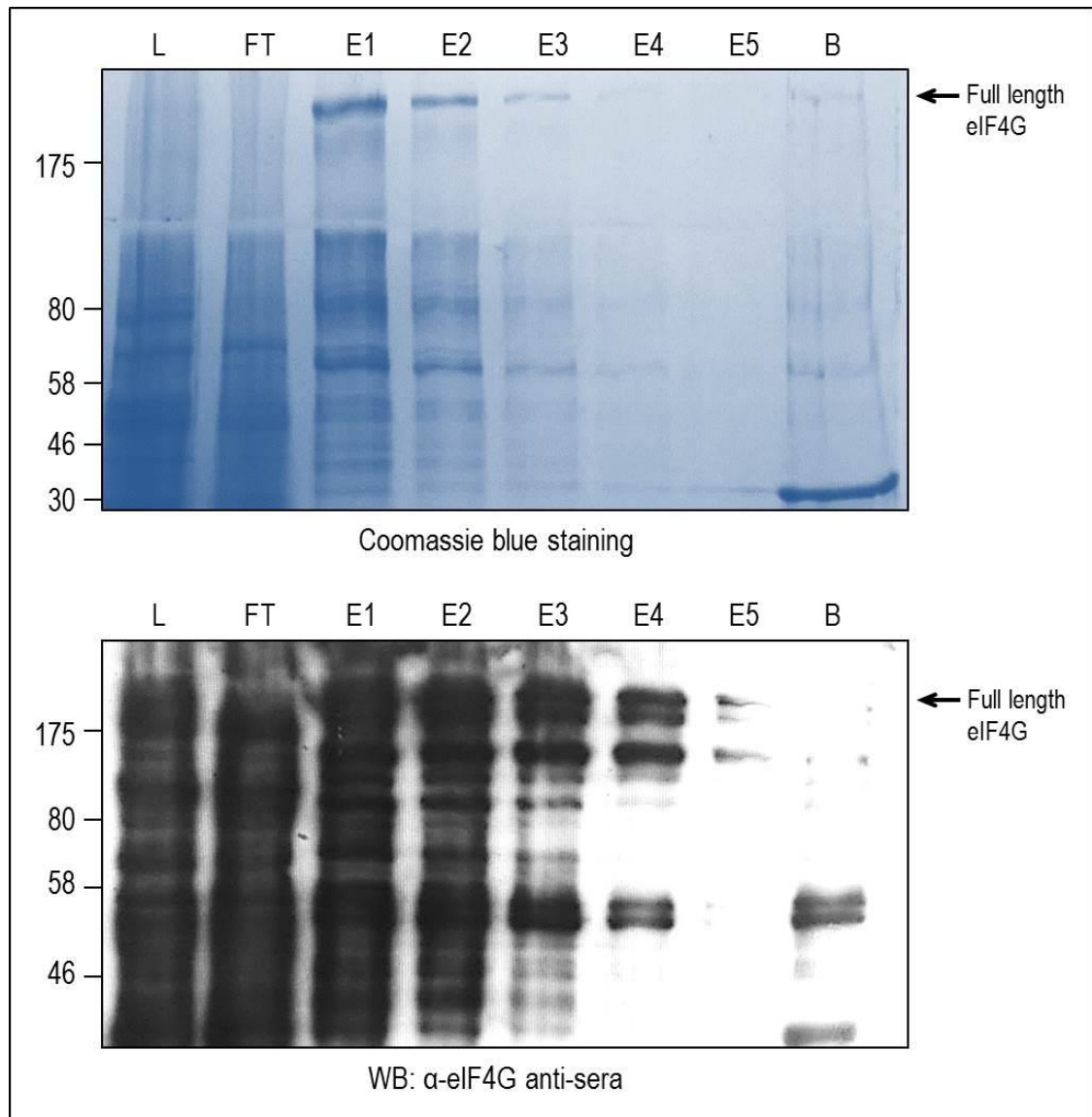


Figure 5.6 Human eIF4G purification using Ni^{2+} agarose beads. Human eIF4G tagged with FLAG and 6xHis epitopes at the N terminus was produced in *Sf9* insect cells using the Baculovirus expression system. The protein concentrations of each fraction (E1–E5) were 3.30, 1.66, 0.31, 0.03, 0.00 mg/ml, respectively. 10 μl of each sample (L=lysate, FT=flow through, E1–E5= the 1st–5th elution fractions and B=beads) was loaded onto a 10% acrylamide gel stained with Coomassie blue (upper part) while another 10 μl of each sample was loaded onto a gel for western blotting against rabbit anti-eIF4G antisera (lower part).

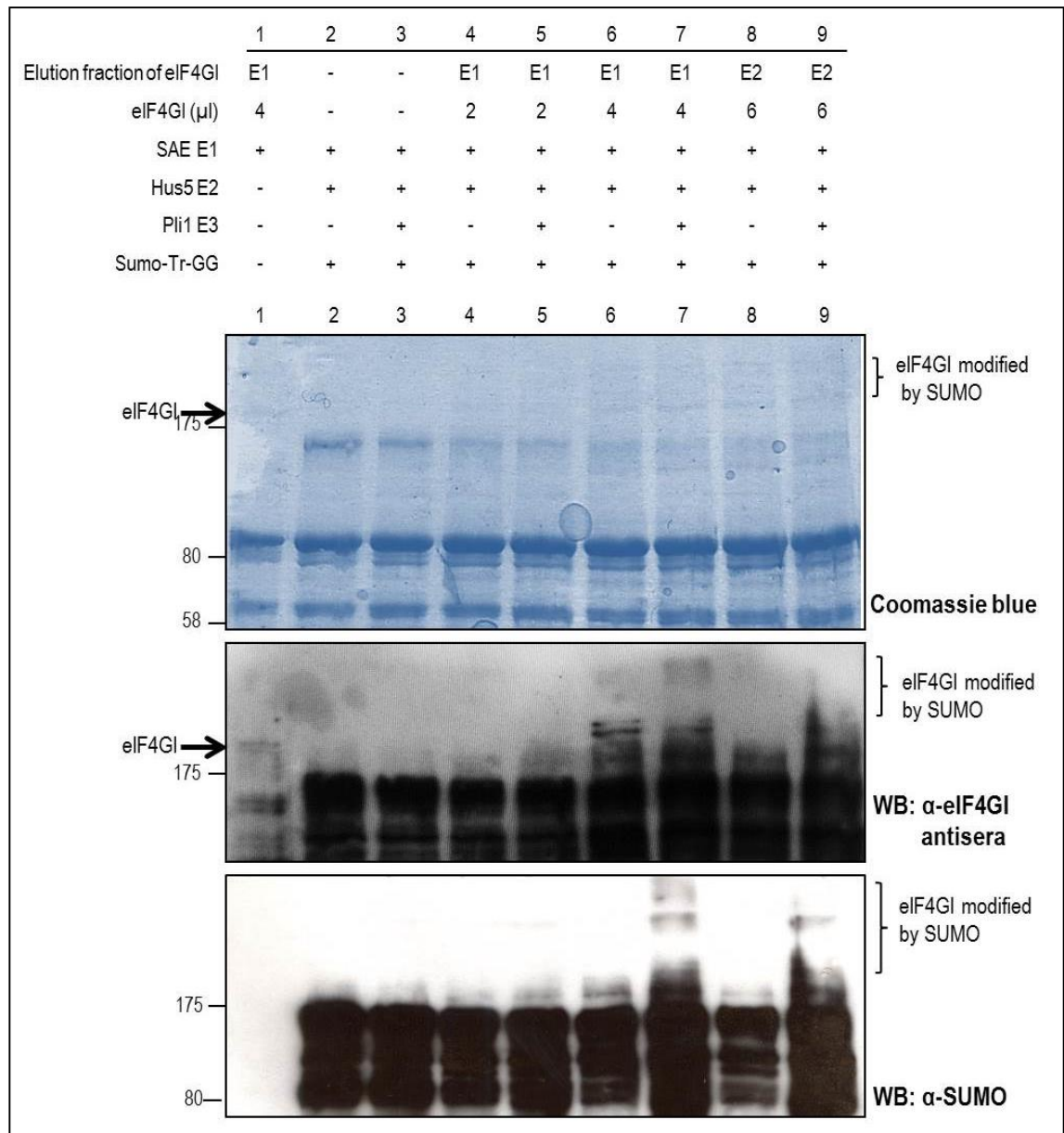


Figure 5.7 *In vitro* SUMOylation of human eIF4G. Full-length human eIF4G produced in *Sf9* insect cells was used in an *in vitro* SUMOylation assay. 6.6 μg of eIF4G fraction 1 (E1) was added in lanes 4–5 and 13.2 μg in lanes 6–7 while 9.9 μg of fraction 2 (E2) was loaded in lanes 8–9. Other components were used in the same concentration as shown in table 5.1. This figure shows staining with Coomassie blue, western blotting (WB) with rabbit anti-eIF4G and anti-SUMO antisera.

5.3.3 Mass spectrometry was unable to identify the SUMOylation sites of full length human eIF4G expressed in the insect cells

Mass spectrometry was undertaken in order to identify which lysine residues are used for SUMOylation. Protein species (over 175 kDa) were excised from lanes 6–9

(from the Coomassie blue-stained gel in Figure 5.7). Proteins contained in the gel pieces were digested with trypsin as described in section 2.5.11. However, using full length eIF4G expressed in *S9* insect cells as a protein target, it was not possible to identify the modified lysine(s) with high confidence by mass spectrometry. This may be due to the large size of full length eIF4G or because SUMOylation levels were low, making the amount of modified peptides insufficient for detection by mass spectrometry. It could also be due to the fact that the SUMOylated lysine residues are present on a very large tryptic peptide that cannot be detected by mass spectrometry.

5.4 Human eIF4G expressed in *E. coli* is SUMOylated *in vitro*

5.4.1 Expression and purification of human eIF4G in *E. coli* system

To further investigate the SUMOylation *in vitro* of eIF4G, eIF4G was expressed in three truncated forms, an N-terminal fragment from 1 to 532 (N-FAG), a middle fragment from 533 to 1176 (M-FAG) and a C-terminal fragment from 1177 to 1600 (C-FAG). These three fragments of eIF4G relate to the caspase-3-mediated cleavage fragments produced during apoptosis (Figure 1.3), called as the apoptotic cleavage fragments of eIF4G (FAG). The map of each fragment is shown in Figure 5.8. The ORF of each fragment was sub-cloned in pET28b (Prof Simon Morley, Sussex). Thus, these proteins could be expressed as N-terminally His-tagged fusion proteins.

BL21 (DE3) *E. coli* cells were transformed with pET28b containing ORF encoding human 6xHis-tagged N-FAG, M-FAG and C-FAG eIF4G. His-tagged fusion proteins (from a one litre culture) were expressed as previously described (see section 2.5.12), and cells were harvested for isolation of His-tagged recombinant proteins using Ni²⁺ agarose beads (described in 2.5.13). The concentration of each elution was analysed using a Bradford assay. To check the purity of recombinant protein, 10 µl of lysate (L), flow through (FT) and each elution (E1–E5) were loaded on a 10% acrylamide gel for Coomassie blue staining. Figure 5.9 shows a good yield of C-FAG of eIF4G (purified by D. Gunton, final year project student, Sussex). Other purified fragments had a lower yield likely because they are present as insoluble protein or in inclusion bodies. However, there was enough of each fragment of eIF4G to use in *in vitro* SUMOylation assays.

5.4.2 *In vitro* SUMOylation analysis of C-FAG eIF4G produced in *E. coli*

The three fragments of human eIF4G (N-, M- and C-FAG) expressed in *E. coli* were tested in the *in vitro* SUMOylation assay. 6xHis tagged C-FAG eIF4G contains an N-terminal His epitope and the C-terminal eIF4G region of 424 amino acids between 1177 and 1600. The expected size is around 50 kDa. The amount of protein target was varied in the SUMOylation reaction *in vitro*. As shown in Figure 5.10, 4 µg of C-FAG fraction 4 used in lane 1 (control lane), 15 µg from elution fraction 3 (used in lanes 6, 7) and 10 µg from elution 4 (used in lane 8, 9) were added in the reaction according to amount shown in Table 5.1. The extra high molecular weight species in lanes 6–9 are between 58 kDa and 80 kDa. Theoretically, protein targets modified by a single SUMO normally are shifted by approximately 20 kDa. This result suggests that the C-terminal fragment of eIF4G (C-FAG) is SUMOylated *in vitro*. Using similar SUMOylation assays, N-FAG and M-FAG were not found to be SUMO modified (data not shown). The remainder of the section therefore focusses on the analysis of the SUMOylation sites in C-FAG.

5.4.3 Mass spectrometry shows two lysines of eIF4G SUMOylated in C-terminal region

To identify the lysine residues modified by SUMO, the bands corresponding in size to those expected to be C-FAG modified by SUMO-Tr-GG, were excised from the gel and processed for analysis by mass spectrometry as previously described in section 2.5.11. The results of mass spectrometry confirmed that C-FAG is SUMOylated. Mass spectra identify with high confidence two lysines attached to diglycine (GG): these are K1386 and K1588 (Figure 5.11 A and B). Interestingly, the former lysine is located in the eIF4A binding region while the latter lysine is in the Mnk binding region (figure 5.11 C). Figure 5.12 indicates that it should be accessible for SUMOylation.

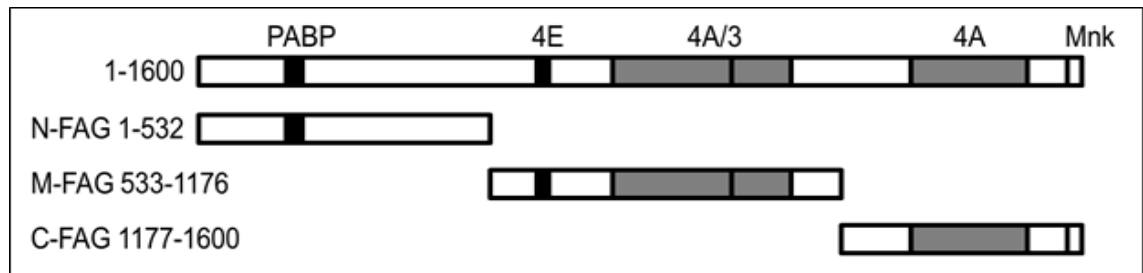


Figure 5.8 Fragments of human eIF4G expressed in *E. coli* are used for the *in vitro* SUMOylation assay. Schematic structures of eIF4G proteins showing the sequences used for expression in this study. This figure also indicates regions involved in the interaction with other proteins (PABP= polyA binding protein, 4E=eIF4E, 4A=eIF4A, 3=eIF3 and Mnk=MAP kinase-interacting kinase 1).

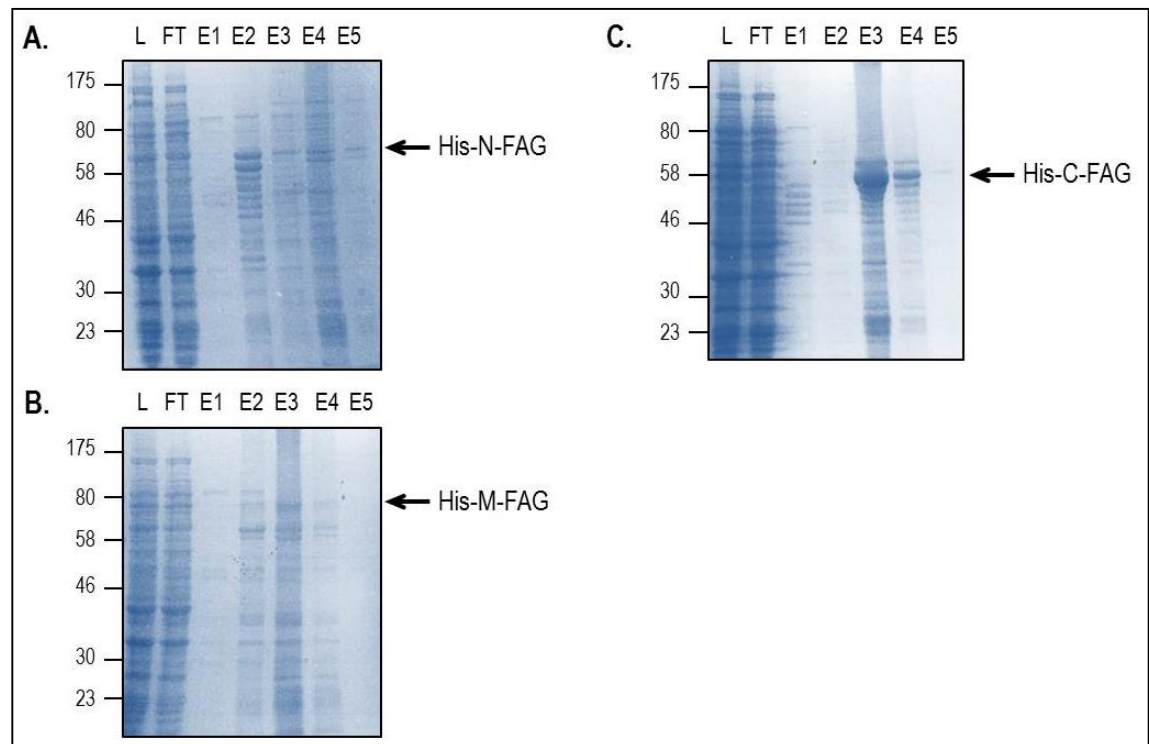


Figure 5.9 Coomassie blue staining of 12.5% acrylamide gels containing His-tagged proteins purified. 10 μ l each elution fraction of A) His-tagged N-FAG, B) His-tagged M-FAG and C) His-tagged C-FAG was loaded onto the individual acrylamide gels to stain with Coomassie blue. (L= lysate, FT= the first flow through, E1-E5= the elution 1st-5th).

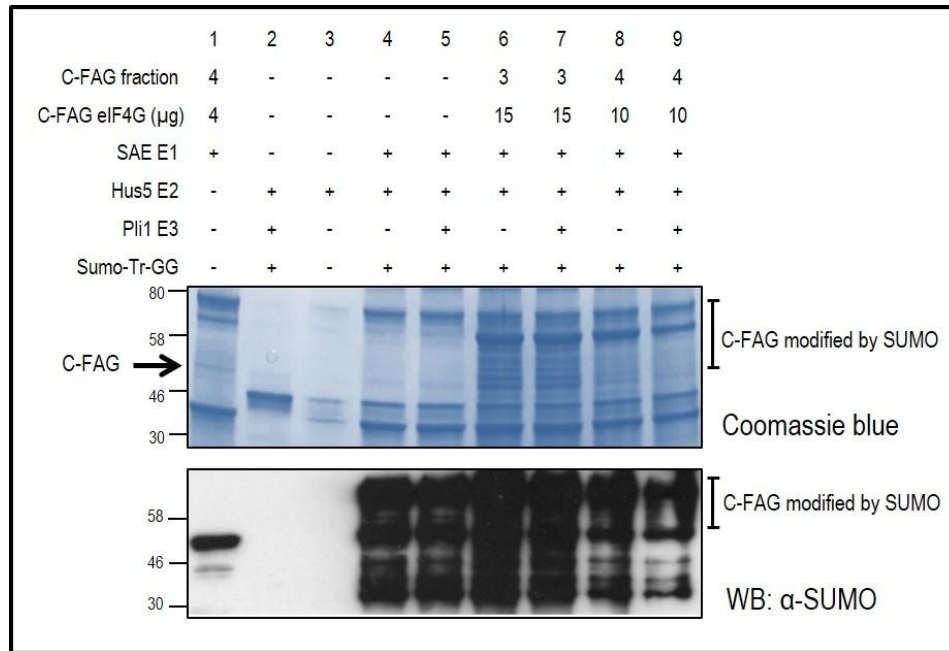


Figure 5.10 The C-FAG of human eIF4G could be SUMOylated *in vitro*. The figures show the effect of adding SUMO components in each reaction and amount of C-FAG. The expected size of C-FAG is around 50 kDa. The extra high molecular weight species in lane 6–9 being between the 58 kDa and 80 kDa, were excised to identify SUMOylation sites of C-FAG eIF4G by mass spectrometry.

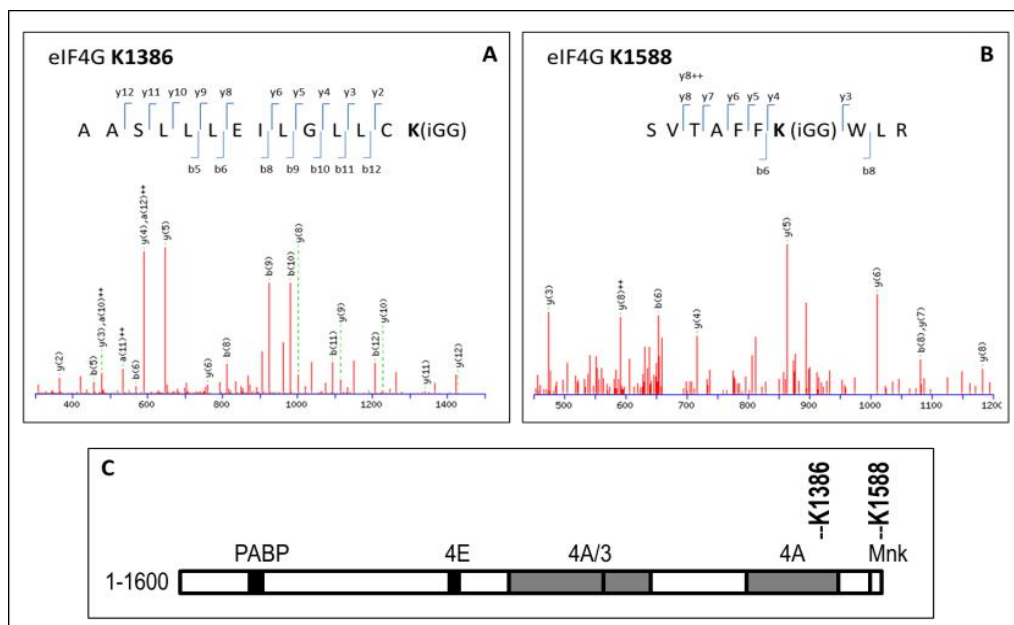


Figure 5.11 Mass spectrophotometry identified two SUMOylation sites on C-FAG. A. and B. Mass Spectra of eIF4G peptides modified by GG at K1386 and K1588, respectively and C. Schematic diagram of eIF4G indicating position of SUMOylated lysines.

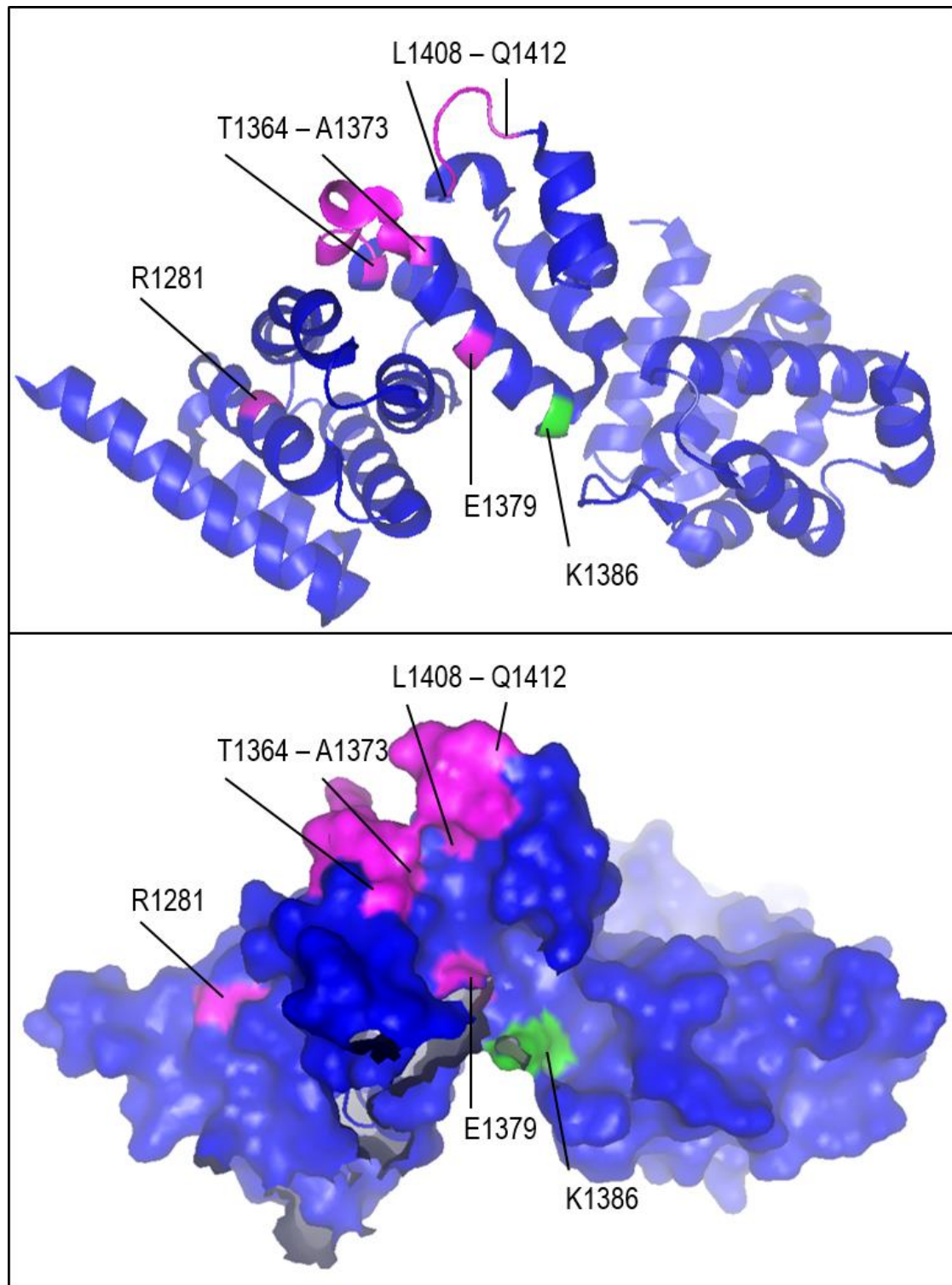


Figure 5.12 Crystal structures of C-terminal portion of eIF4GI. This figure indicates amino acid residues between 1234 and 1572 (PDB ID 1UG3). Thus only K1386 (green) modified by SUMO is shown in the C-terminal structure (blue) while K1588 is not shown in the structure (Bellsollell *et al.*, 2006). Residues in magenta are eIF4A binding sites on eIF4GI which are next to Lysine 1386, suggesting that SUMOylation of eIF4G on K1386 may affect to the interaction of eIF4G and eIF4A.

5.5 SUMOylation of human eIF4G *in vivo*

To determine whether human eIF4G is SUMOylated *in vivo*, two main approaches were taken. The first was affinity purification of His-tagged SUMO expressed in mammalian cell lines using Ni²⁺ agarose beads, and the second was alteration of eIF4G SUMOylation levels in response to stresses.

5.5.1 Human eIF4G is SUMOylated *in vivo* confirmed by Ni²⁺ affinity purification

HeLa cells that stably express 6xHis-tagged SUMO1 (S1) or 6xHis-tagged SUMO2 (S2) (a kind gift from Prof Ron Hay, University of Dundee), were used in order to investigate SUMOylation of human eIF4G *in vivo*. Non-transfected HeLa cells (NT) were used as a negative control. Cell lysates were prepared under denaturing conditions to improve the yield of SUMOylated species as previously described in section 2.5.19. His-tagged SUMO1 and SUMO2 were affinity purified from the cell extracts by Ni²⁺ agarose beads (Figure 5.13 Panels C and D). It has previously been demonstrated that eIF4E is modified by SUMO (Xu *et al.*, 2010). Figure 5.13 (Panel B) shows that, under conditions used here, eIF4E is pulled down with both His-SUMO1 and His-SUMO2, confirming that eIF4E is SUMOylated. Figure 5.13 (Panel A) indicates that eIF4G is not recovered from extracts of cells that do not contain His-tagged SUMO (in PD, NT lane), but is isolated from extracts of cells containing His-SUMO1 (in PD, S1 lane) and to a lesser extent from cells expressing His-SUMO2 (in PD, S2 lane). This confirms that, like *S. pombe* eIF4G, human eIF4G is SUMOylated.

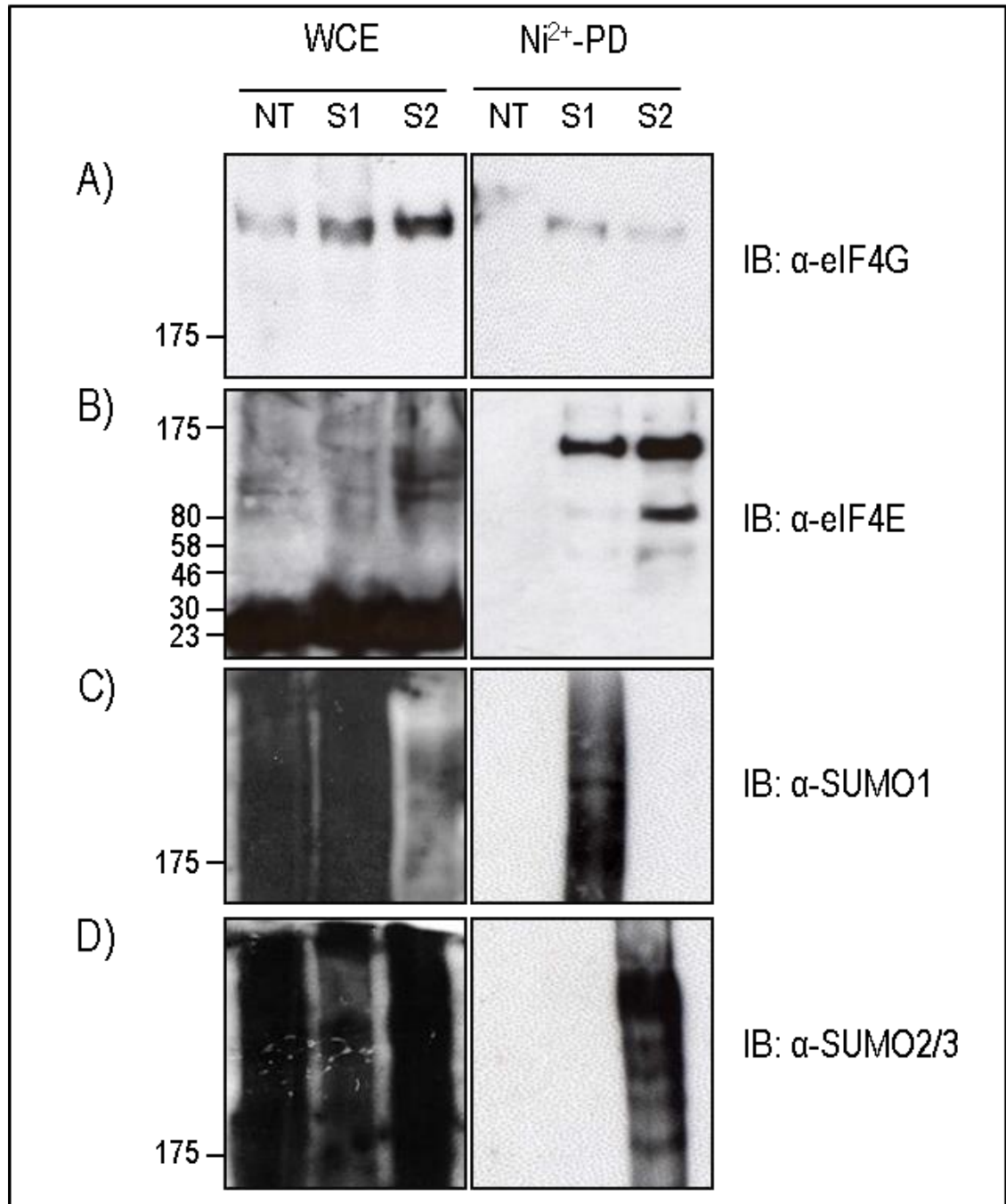


Figure 5.13 Western blotting of Ni²⁺ affinity purification of eIF4G and eIF4E. Whole cell extraction (WCE) and Ni²⁺ affinity purification (PD) from HeLa cells, non-transfected (NT) or transfected of either His-tagged SUMO1 or SUMO2 were analysed on 7.5% PAGE. Blots were probed with anti-eIF4G (A), anti-SUMO1 (C) and anti-SUMO2/3 (D) antibodies and blots from 12.5% PAGE was probed with anti-eIF4E antisera (B). The results indicate that eIF4G is modified by both SUMO1 and SUMO2. eIF4E was included as a positive control.

5.5.2 The levels of eIF4G modified by SUMO1 is altered in response to ionising radiation

HeLa cells containing either His-SUMO1 or His-SUMO2 grown to 60–80% confluence in T175 flasks, were treated with or without either 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). His-tagged SUMO1 and SUMO2 were affinity purified from cells extracts using Ni^{2+} agarose beads under denaturing conditions as described in section 2.5.19, and compared to the negative control which was HeLa cells untreated (UT). Whole cell extracts (WCE) and affinity purify (PD) samples were loaded onto 7.5% acrylamide gels. Western blots were probed with anti-eIF4G antibody. Figure 5.14 shows that eIF4G was recovered from cell extracts that contain His-tagged SUMO1 (lanes 2–4) and to a lesser extent from extract containing His-tagged SUMO2 (lane 5–7). This confirms that eIF4G is modified by SUMO1 and possibly SUMO2. Interestingly, the levels of eIF4G modified by SUMO1 are decreased in response to ionising radiation. Figure 5.14 shows that there are degraded species of eIF4G which are also affinity purified with SUMO1 and SUMO2. These may be SUMOylated species of eIF4G. Thus it is possible that eIF4G (small molecular weight species) modified by SUMO2 are increased in response to arsenite. Nevertheless, the amount of eIF4G modified by SUMO2 is lower than that of eIF4G modified by SUMO1 which is consistent with Figure 5.13.

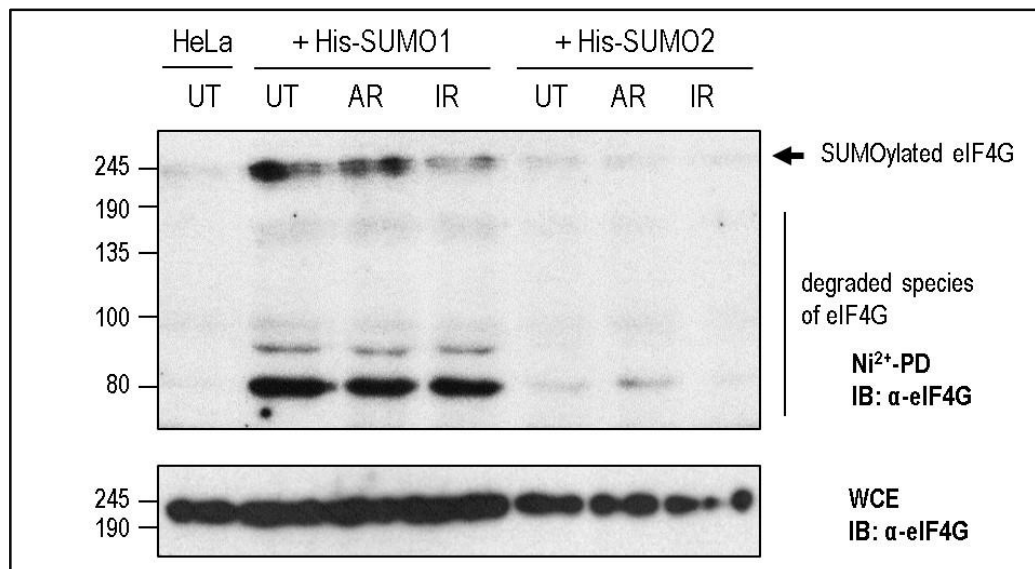


Figure 5.14 Analysis of SUMOylation of eIF4G in response to stresses. Whole cell extracts (WCE) and Ni^{2+} pulled-downs from wild type HeLa cells and HeLa cells stably transfected with His-tagged SUMO1 or SUMO2 were loaded onto 7.5% gels. Western blots were probed with anti-eIF4G antisera.

5.6 Immunofluorescence analysis of human eIF4G and SUMO1

Because eIF4G is modified by SUMO1, it was of interest to determine whether eIF4G and SUMO1 colocalise. HeLa cells were therefore grown to 60–80% confluence on cover slides, and then left untreated or treated with either 1 mM sodium arsenite or 3 Gy ionising radiation. For microscopy, untreated (UT) and treated cells (AR and IR) cells were incubated with two primary antibodies, namely mouse anti-eIF4G and rabbit anti-SUMO1. FITC anti-mouse IgG and Cy3 anti-rabbit IgG were used against the specific primary antibodies as described in section 2.4.8. eIF4E was used as a control.

The immunofluorescence result (Figure 5.15) shows that eIF4G (green) is mainly present in the cytoplasm whereas SUMO1 (red) is mainly in the nucleus, although there is a small amount in the cytoplasm. In untreated cells there is a low level of colocalisation of eIF4G and SUMO1 in both the nucleus and the cytoplasm (Figure 5.16). Interestingly, in arsenite-treated cells most of the eIF4G is observed in stress granules in the cytoplasm. At many of the stress granules SUMO1 is found at the edges (Figure 5.17). In IR-treated cells, SUMO1 is mainly in foci in the nucleus. In these HeLa cells, there is low, but significant level of colocalisation of eIF4G with SUMO1 in the nucleus (Figure 5.18). Thus, colocalisation of eIF4G and SUMO1 is increased in cells exposed to both arsenite and ionising radiation.

As it is known that eIF4E is SUMOylated (Xu *et al.*, 2010), HeLa cells were analysed for the colocalisation of eIF4E and SUMO1 to see how this compared to the amount of colocalisation observed for eIF4G and SUMO1. Figure 5.19 shows that the result is similar to that of eIF4G. For example, in untreated cells there is a low level of colocalisation of eIF4E with SUMO1 in both the nucleus and the cytoplasm (Figure 5.20). In arsenite-treated cells most of the eIF4E is observed in stress granules in the cytoplasm. At many of the stress granules SUMO1 is found at the edges (Figure 5.21). In IR-treated cells, SUMO1 forms more foci in the nucleus and some in the cytoplasm. In these cells eIF4E colocalises with SUMO1 in the nucleus (Figure 5.22).

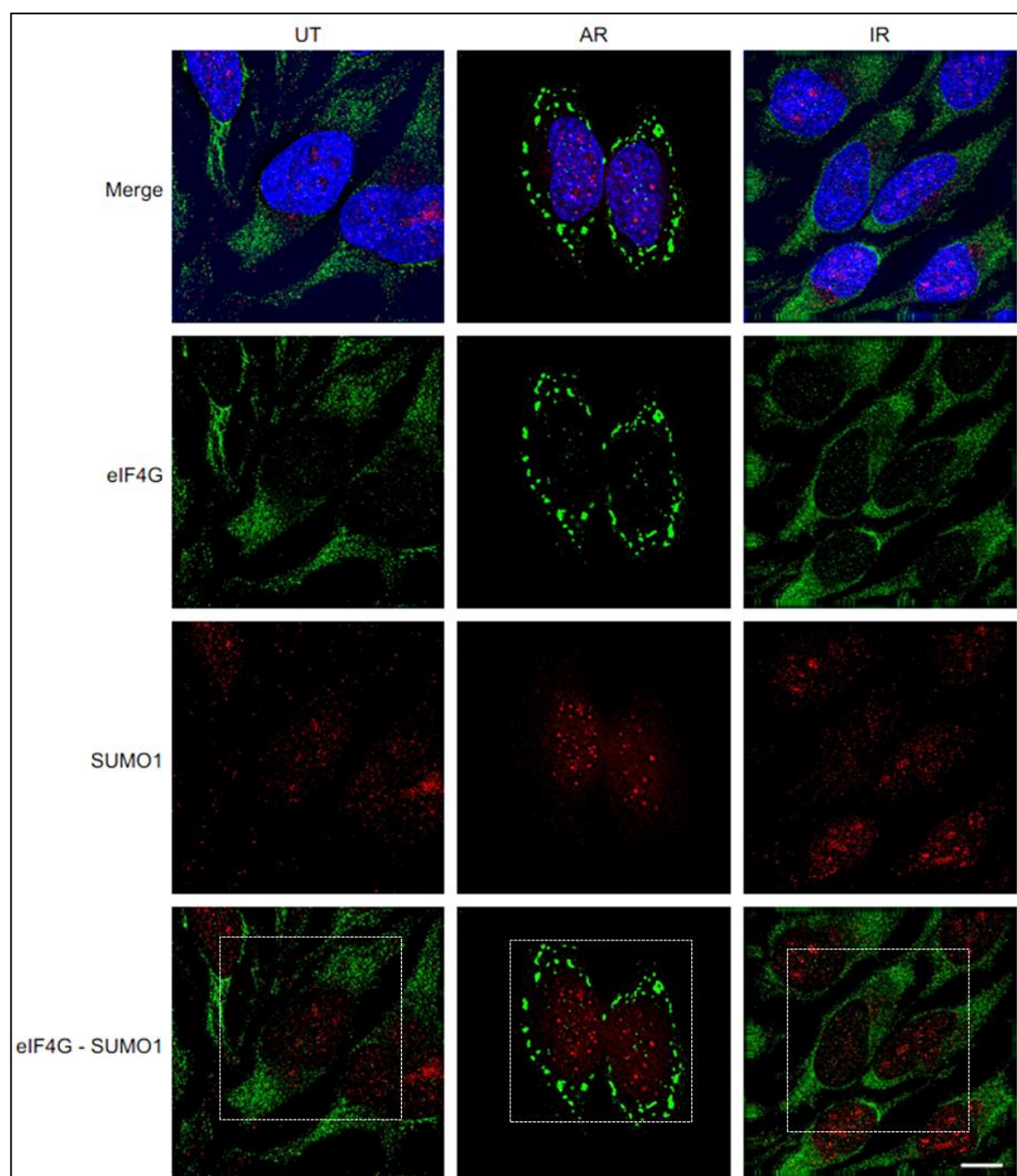


Figure 5.15 Immunofluorescence indicating colocalisation of eIF4G and SUMO1. HeLa cells untreated (UT) and treated with 1 mM sodium arsenite (AR) or 3 Gy gamma source (IR). Blue, green and red colours represent the DAPI-stained DNA, eIF4G and SUMO1, respectively. The bar scale is 10 μ m. The white boxes are shown in the expanded versions as Figure 5.16–5.18.

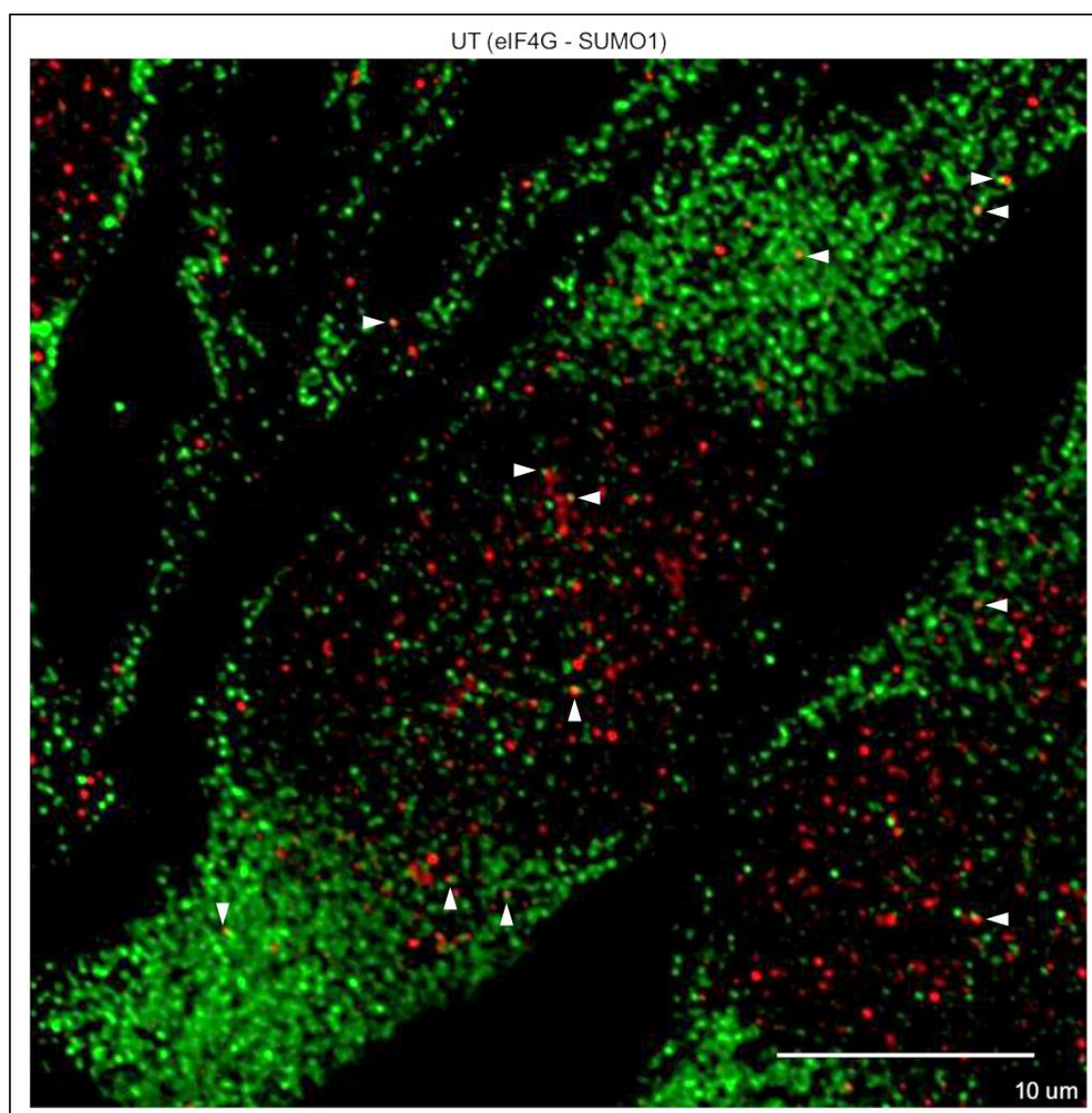


Figure 5.16 Expanded version from Figure 5.15. Immunofluorescence of HeLa cells untreated (UT). Green and red colours represent eIF4G and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4G and SUMO1.

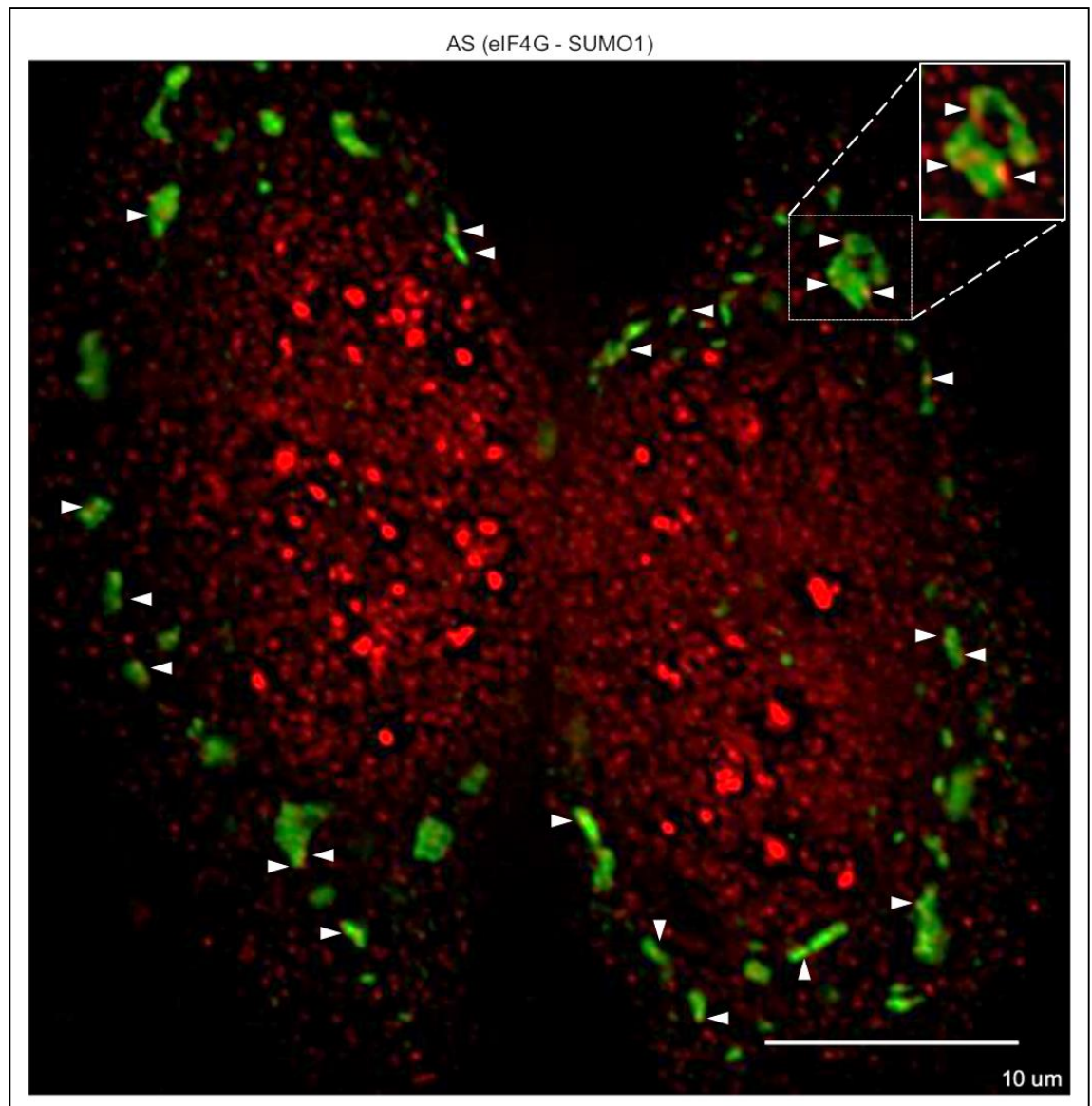


Figure 5.17 Expanded version from Figure 5.15. Immunofluorescence of HeLa cells treated with 1 mM sodium arsenite (AR). Green and red colours represent eIF4G and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4G and SUMO1. The small box shows the colocalisation of SUMO1 and eIF4G at the edges of stress granules, and this is shown by adjusting higher intensity of red and lower intensity of green for presenting the clearer versions of the figure only.

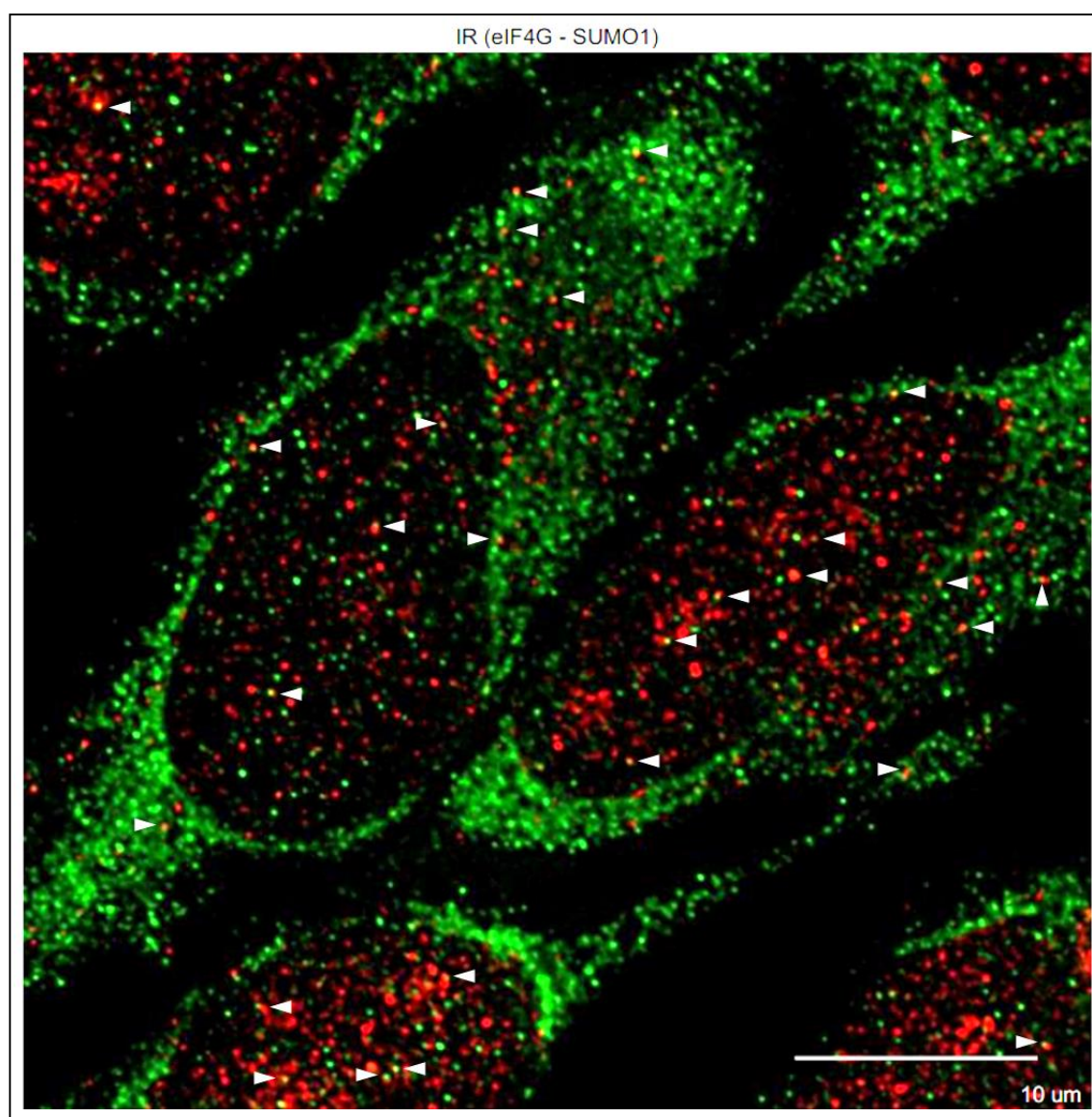


Figure 5.18 Expanded version from Figure 5.15. Immunofluorescence of HeLa cells treated with 3 Gy ionising radiation (IR). Green and red colours represent eIF4G and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4G and SUMO1.

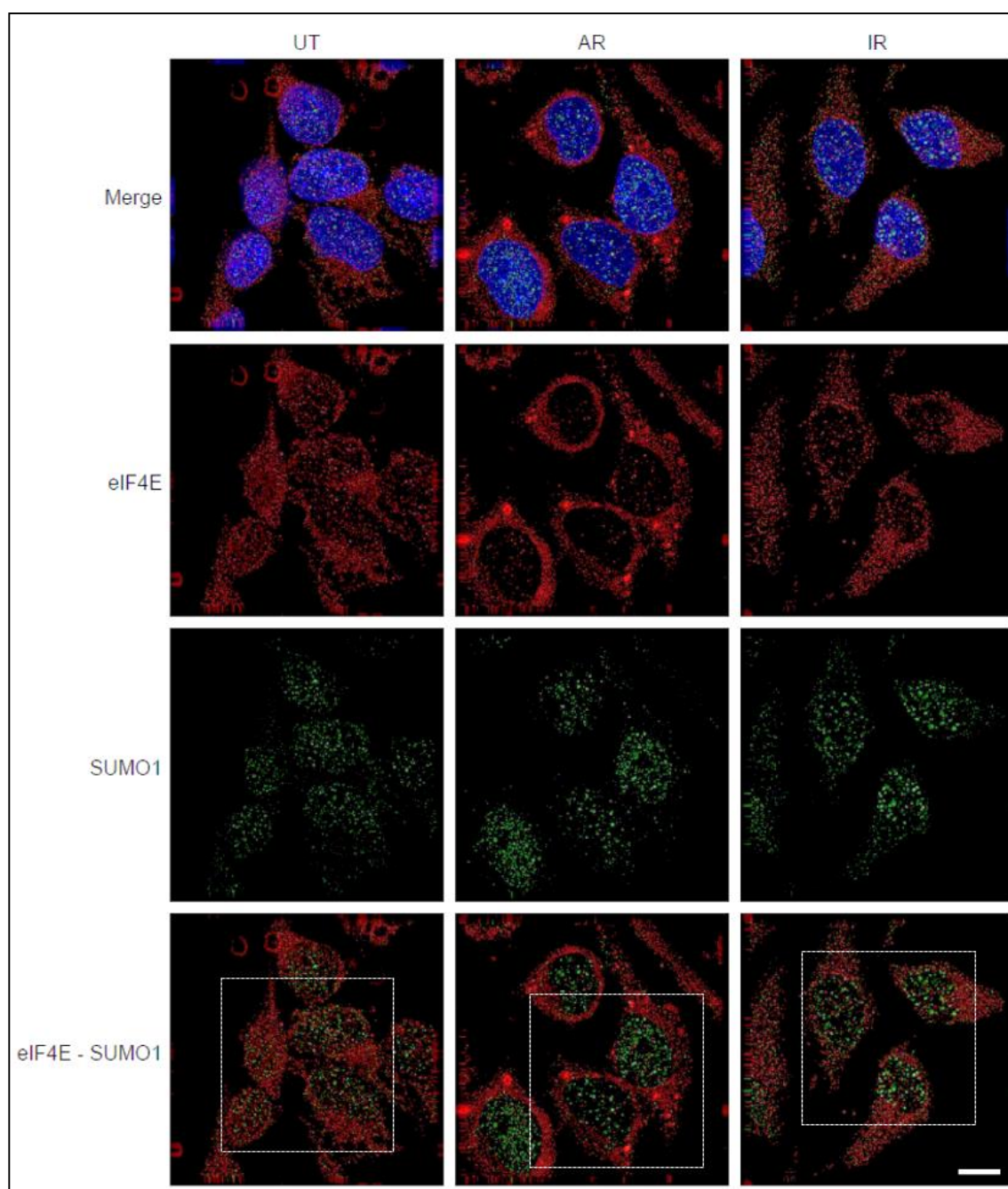


Figure 5.19 Immunofluorescence indicating colocalisation of eIF4E and SUMO1. HeLa cells untreated (UT) and treated with 1 mM sodium arsenite (AR) or 3 Gy gamma source (IR). Blue, red and green colours represent the DAPI-stained DNA, eIF4E and SUMO1, respectively. The bar scale is 10 μm . The white boxes are shown in the expanded versions as Figure 5.20–5.22.

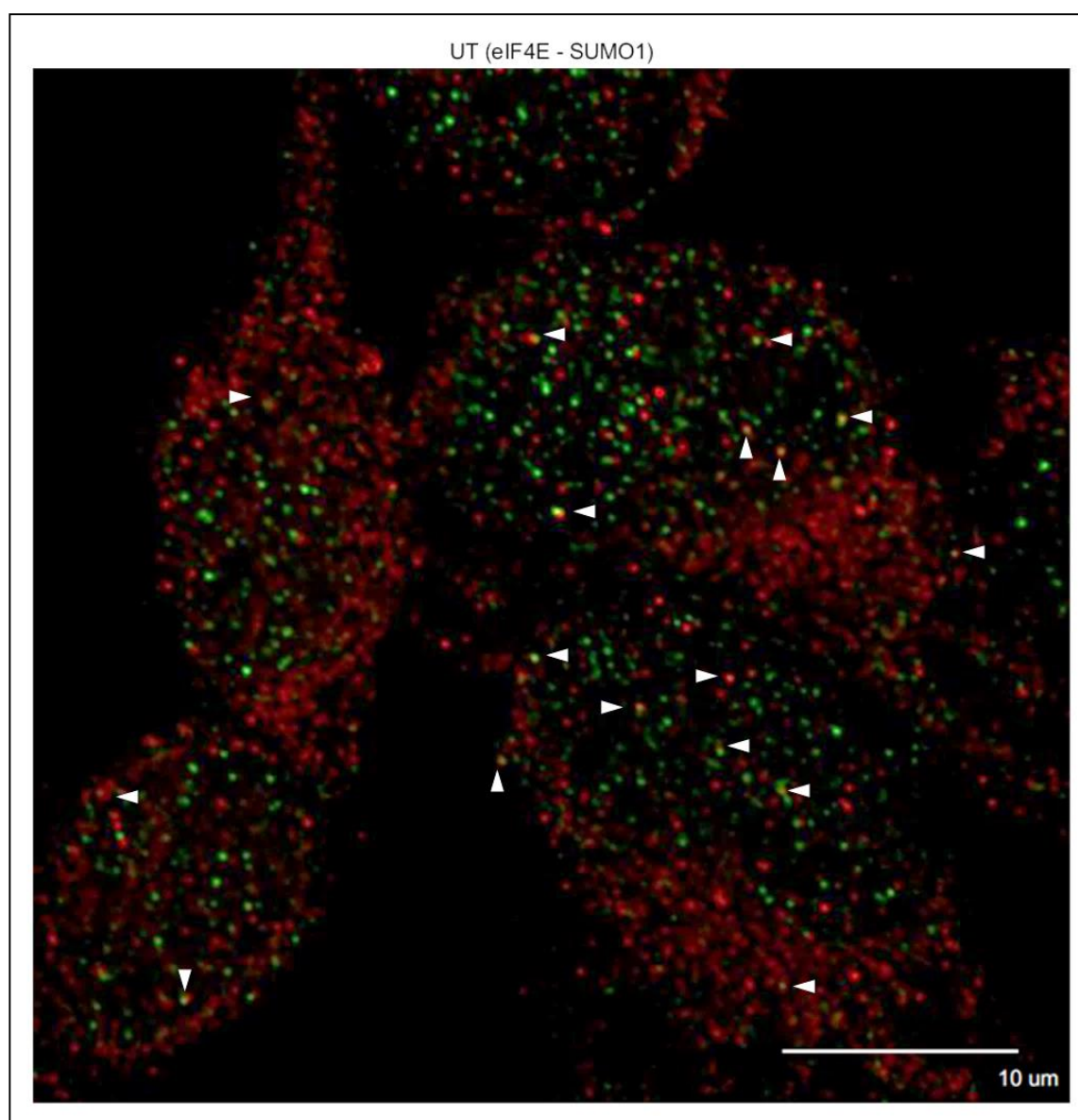


Figure 5.20 Expanded version from Figure 5.19. Immunofluorescence analysis of untreated HeLa cells. Red and green colours represent eIF4E and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4E and SUMO1.

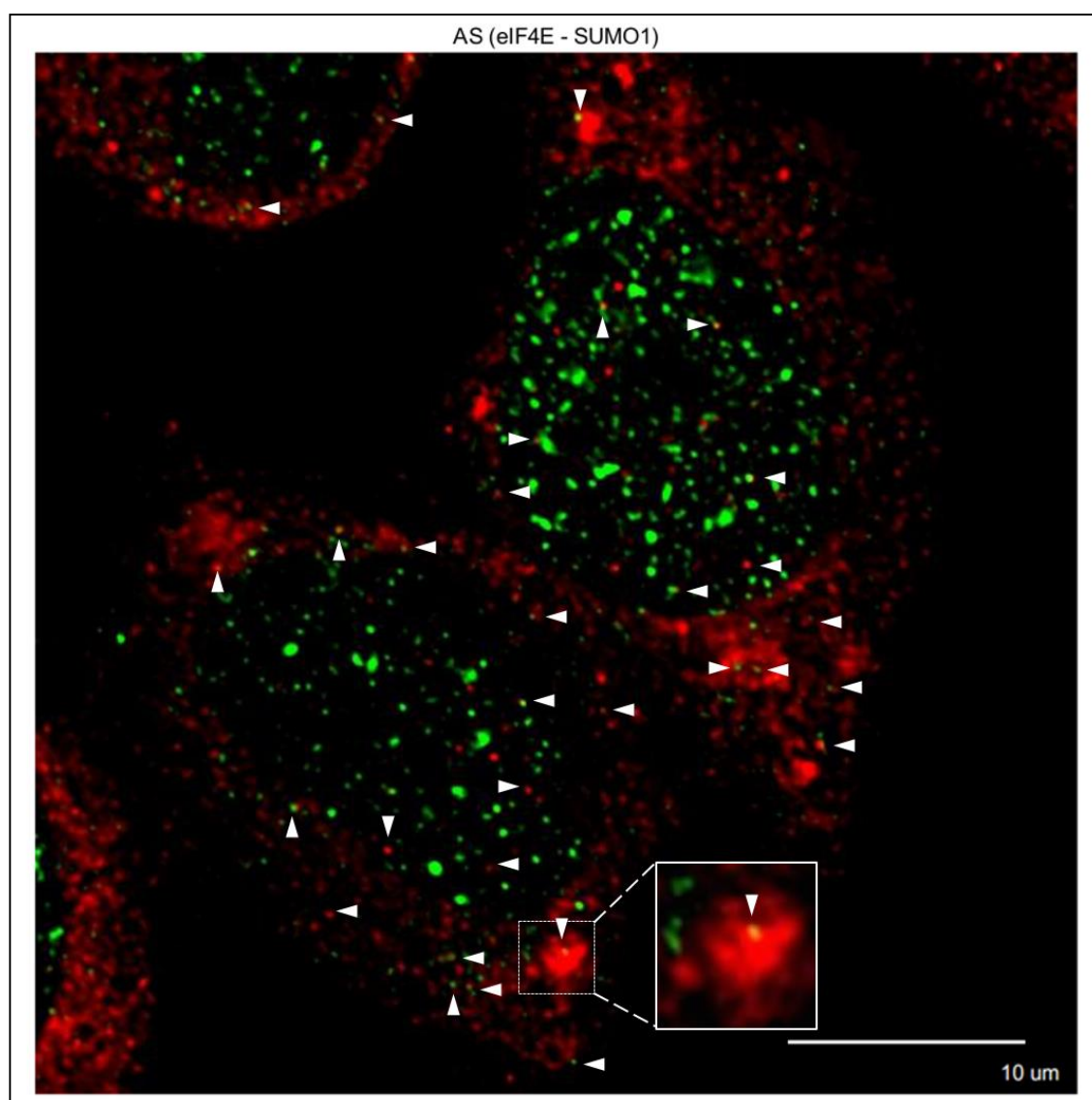


Figure 5.21 Expanded version from Figure 5.19. Immunofluorescence analysis of sodium arsenite-treated HeLa cells. Red and green colours represent eIF4E and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4E and SUMO1. The small box shows the colocalisation of SUMO1 and eIF4E at the stress granules.

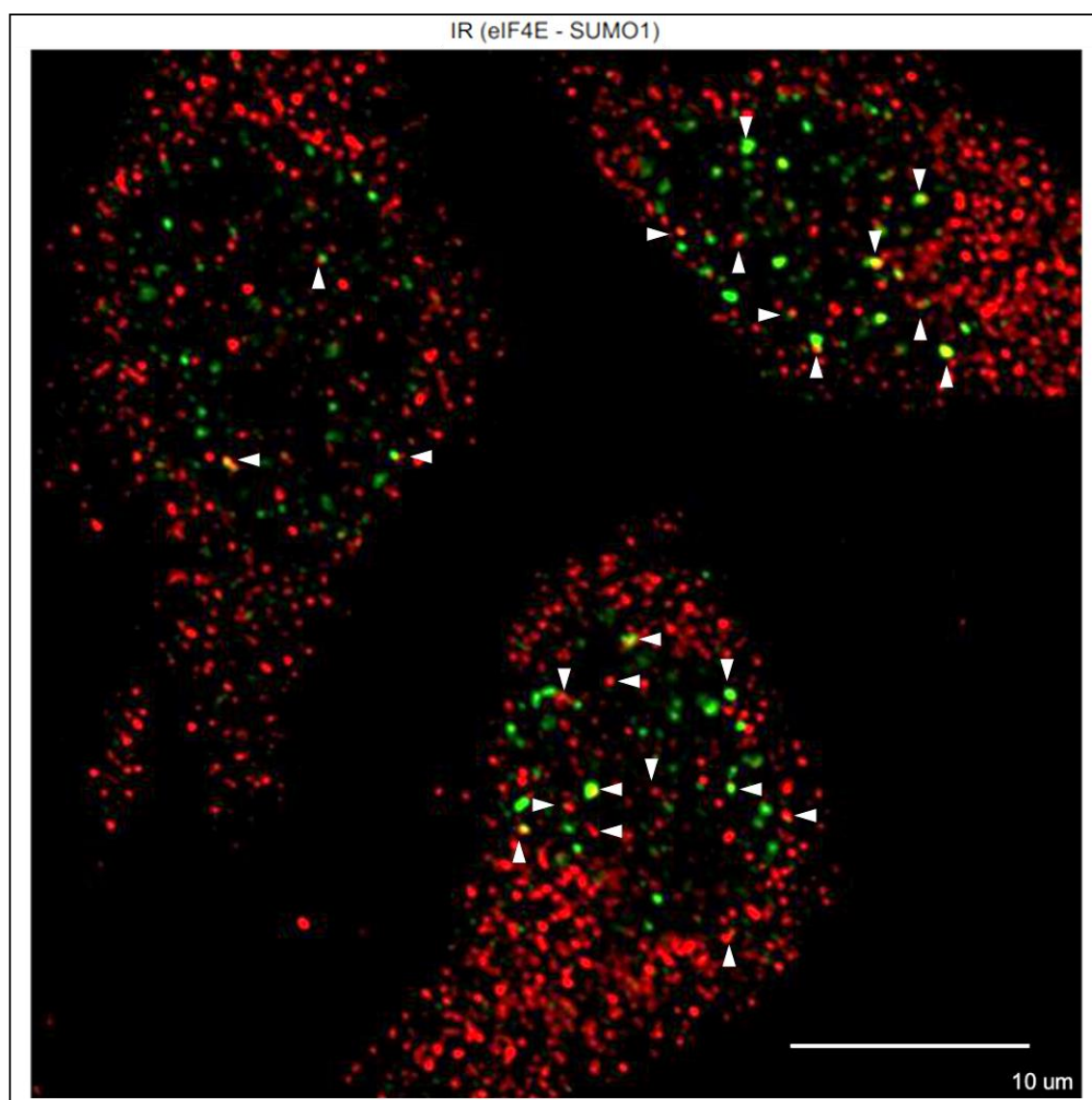


Figure 5.22 Expanded version from Figure 5.19. Immunofluorescence analysis of IR-treated HeLa cells. Red and green colours represent eIF4E and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4E and SUMO1.

5.7 Discussion

As an *in vitro* SUMOylation assay of protein targets has been previously available in our laboratory (Ho *et al.*, 2001), it was first used to analyse SUMOylation of eIF4G *in vitro*. The recombinant SUMOylation components of the assay were prepared here and were analysed for ability to form SUMO chains using the conditions adapted from Ho *et al.* (Ho *et al.*, 2001). The amount of each component used is shown in Table 5.1. All components were capable of forming SUMO chains in an *in vitro* SUMOylation assay.

Human eIF4G was assayed for ability to be SUMOylated *in vitro*. The human protein was purified from two different sources. One was *Sf9* insect cells using the Baculovirus system and another was *E. coli*. Human full length eIF4G purified from insect cells was shown to be SUMOylated *in vitro*, particularly in the presence of Plp1 (Figure 5.7), suggesting that human eIF4G may be SUMOylated through a SUMO E3 ligase-dependent pathway. For the identification of SUMOylation sites of eIF4G, using full length eIF4G expressed in *Sf9* insect cells as a protein target, it was not possible to identify the modified lysine(s) with high confidence by mass spectrometry. This may be due to the large size of full length eIF4G or because SUMOylation levels were low, making the amount of modified peptides insufficient for detection by mass spectrometry. It could also be due to the fact that the SUMOylated lysine residue(s) is/are present on a very large tryptic peptide that cannot be detected by mass spectrometry.

Using an alternative approach, three truncated recombinant proteins were expressed in *E. coli*, namely an N-terminal fragment (N-FAG 1–532), a middle fragment (M-FAG 533–1176) and a C-terminal fragment (C-FAG 1177–1600) (Figure 5.8), then tested in the *in vitro* SUMOylation assay (Figure 5.10). Only C-FAG was observed to be modified by SUMO *in vitro* while N-FAG and M-FAG were not. Mass spectrometric analysis (Figure 5.11) confirmed that C-FAG is SUMOylated with high confidence on two lysines attached to diglycine (GG). These are K1386 and K1588. Interestingly, the former lysine is located in the HEAT2 domain while support the second eIF4A binding region while the latter lysine is in the HEAT3 domain which supports Mnk1 binding region and (Figure 5.12) (Coldwell *et al.*, 2004). K1386 on eIF4G is not in the eIF4A contact surface but it is very close to the area (Marintchev *et al.*, 2009). It is possible that SUMOylation of eIF4G may change eIF4G conformation which could possibly provide

the altered surface in order to interact with eIF4A or interfere the eIF4A binding. Likewise, SUMOylation of K1588 on eIF4G may have a role in the binding with Mnk1.

In order to verify that eIF4G is indeed SUMOylated, it was necessary to demonstrate SUMOylation *in vivo* (Figure 5.13). Human 6xHis-SUMO1 and 6xHis-SUMO2 were affinity purified using Ni²⁺ agarose beads under denaturing conditions. As a control, eIF4E, which has been previously shown to be SUMOylated (Xu *et al.*, 2010), was pulled down with both SUMO1 and SUMO2. This was also the case for eIF4G which was isolated with SUMO1 and SUMO2. This confirmed that human eIF4G is modified by both SUMO1 and SUMO2. Additionally, in response to IR, the levels of SUMO1-modified eIF4G are slightly decreased (Figure 5.14). This suggests that both AR and IR affect the levels of SUMOylation of eIF4G.

I had several attempts to scale up and purify the SUMOylated forms of endogenous eIF4G for mass spectrometry in order to identify the SUMOylation sites used *in vivo*. Unfortunately the SUMOylation sites on eIF4G were unable to be identified using mass spectrometry (data not shown). This may be because trypsin cleavage of endogenous human SUMO produces a large C-terminal fragment (ELGMEEEDVIEVYQEQTGG) for mass spectrometry. This would make it difficult to identify the large mass of wild type human SUMO cleaved by trypsin on SUMOylation sites of endogenous human eIF4G. In further work, this could be solved using human cells containing 6xHis-SUMO (SUMO1 or SUMO2) T105R which would encode an alternative human SUMO protein with a trypsin cleavage site adjacent to the GG motif.

Here, it is shown that human eIF4G is SUMOylated *in vivo* and *in vitro*. Two SUMOylation sites on human eIF4G were identified which are not conserved in fission yeast eIF4G. This is because the *S. pombe* protein does not contain the C-terminal domains appeared in human eIF4G.

Since one of the functions of SUMOylation is to affect protein localisation, eIF4G was investigated for colocalisation with SUMO using IF (Figure 5.15). In untreated cells (Figure 5.16), SUMO1 is normally found in the nucleus whereas eIF4G is mainly present in the cytoplasm although there is a small amount in the nucleus. However, there is a low level of colocalisation of eIF4G and SUMO1 in both the nucleus and the cytoplasm. This suggests that eIF4G may have a role in regulating RNA processing or localisation in the nucleus and translation in the cytoplasm.

Interestingly, in arsenite-treated cells (Figure 5.17) most of the eIF4G is observed in stress granules in the cytoplasm. Colocalisation of eIF4G and SUMO1 is increased in cells exposed to arsenite. At many of the stress granules, SUMO1 is found at the granules' edges where eIF4G colocalise with SUMO1. This suggests that eIF4G and SUMO1 together may have a role in some aspect of translation when cells are exposed to stress conditions. In IR-treated cells (Figure 5.18), SUMO1 is mainly found in foci in the nucleus. Colocalisation of eIF4G and SUMO1 is increased in cells exposed to IR. Levels of colocalisation of eIF4G with SUMO1 were in the nucleus. This suggests that eIF4G may have a role in regulating RNA processing or localisation.

As the amount of the colocalisation of eIF4G and SUMO1 observed in the cells untreated and treated with arsenite and IR is low, compared to their overall levels in cells, colocalisation of eIF4E and SUMO1 was also analysed. This was used to evaluate the significance of the colocalisation of eIF4G and SUMO1 using immunofluorescence. It is found that the amount of the colocalisation of eIF4E and SUMO1 is similar to that of eIF4G and SUMO1. For example, in untreated cells (Figure 5.20) there is a low level of colocalisation of eIF4E with SUMO1 in both the nucleus and the cytoplasm. In arsenite-treated cells (Figure 5.21) most of the eIF4E is observed in stress granules in the cytoplasm. At many of the stress granules SUMO1 is found at the edges. In IR-treated cells (Figure 5.22), SUMO1 forms more foci in the nucleus and some in the cytoplasm. In these cells eIF4E colocalises with SUMO1 in the nucleus. These results indicate that immunofluorescence is a useful tool for seeing the colocalisation between SUMO1 and eIFs.

Chapter 6

Investigation of SUMOylation of Human eIF4A

6.1 Introduction

Both eIF4E and eIF4G are components of the eIF4F complex. This complex also contains eIF4A, a DEAD-box helicase. Since we know that both human eIF4E and eIF4G are modified by SUMO (Xu *et al.*, 2010; Jongjitwimol *et al.*, 2014; Watts *et al.*, 2014), it was of interest to determine whether human eIF4A is also SUMOylated. Human eIF4A has previously been identified as one of the eIFs identified in proteomic screens for SUMOylated proteins (Table 1.5), suggesting that this is possible. Specifically, both human eIF4AI (Blomster *et al.*, 2009; Bruderer *et al.*, 2011) and eIF4AII (Matafora *et al.*, 2009) were identified in these screens, but to date the modification of these factors has not been confirmed. Therefore, this chapter will focus on an investigation into whether human eIF4AI and eIF4AII are modified by SUMO both *in vitro* and *in vivo*. In addition, the localisation of eIF4A and SUMO in cells has also been analysed.

6.2 Human eIF4A proteins are SUMOylated *in vitro*

6.2.1 Expression and purification of human eIF4A proteins from *E. coli*

6xHis-tagged eIF4AI and GST-tagged eIF4AII were expressed in *E. coli* and purified by Robert Baldock (University of Sussex) using Ni²⁺ agarose purification and GST purification, respectively (previously described in section 2.5.13 and 2.5.14). Cells from a one litre of culture were harvested for the isolation of the recombinant protein. The protein concentration of each recombinant protein was determined using Bradford assay. The purity of eIF4As was analysed by SDS PAGE (data not shown). Both recombinant eIF4AI and eIF4AII, which have high concentration (1 and 4 mg/ml, respectively) with a good purity, were stored in glycerol (10% final concentration). Aliquots were frozen at −80°C for further use.

6.2.2 Mass spectrometry identifies the lysines of eIF4A that are SUMOylated

To assay for the ability to be SUMOylated, both purified 6xHis-tagged eIF4AI and GST-tagged eIF4AII were incubated with SUMOylation components in the *in vitro* assay as described in section 2.5.9 (performed by Robert Baldock, University of Sussex). A Coomassie blue-stained gel of eIF4AII is shown in Figure 6.1. To identify the SUMOylation sites using mass spectrometry, proteins were excised from lanes 3–4 in the region shown Figure 6.1 and from a gel separating SUMOylated eIF4AI species (data not shown). Proteins in the gel pieces were digested with trypsin as described in section 2.5.11. Mass spectra in Figure 6.2 show that K225 of eIF4AI and K226 of eIF4AII are modified by the diglycine (GG) motif. The position of the SUMOylated lysines is shown on the structures of both eIF4AI and eIF4AII in Figure 6.3. It is clear that both lysine residues are located on the outer surface of the molecules (Figure 6.4), consistent with them being available for SUMOylation.

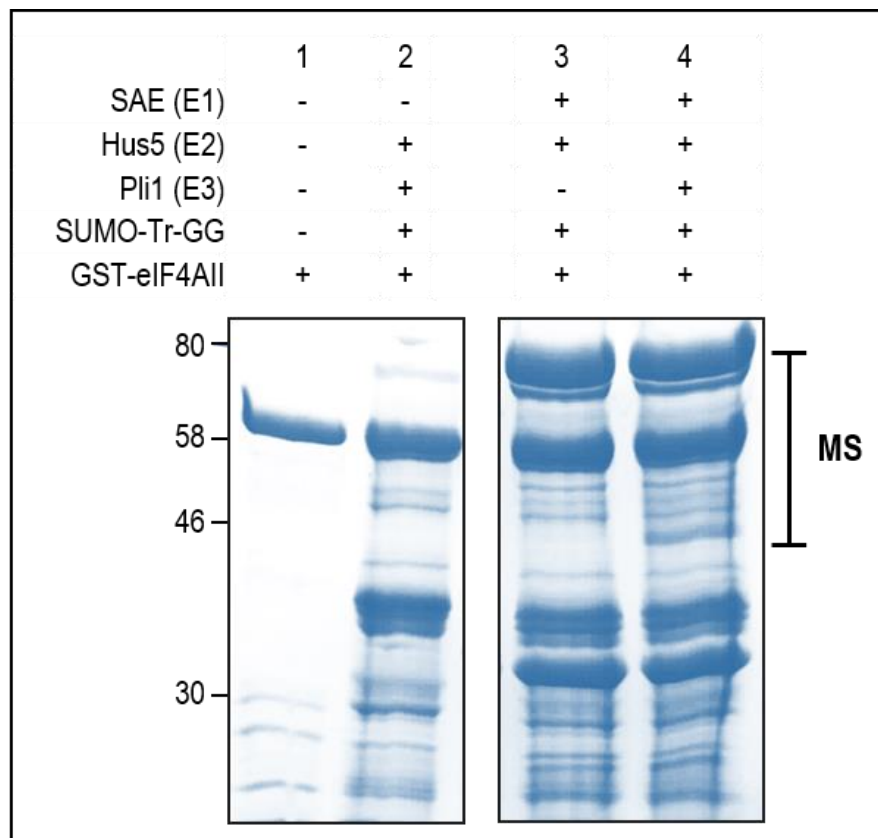


Figure 6.1 An *in vitro* SUMOylation of human eIF4AII. This figure shows a gel stained with Coomassie blue and indicates the region excised for mass spectrometric analysis (R. Baldock, University of Sussex).

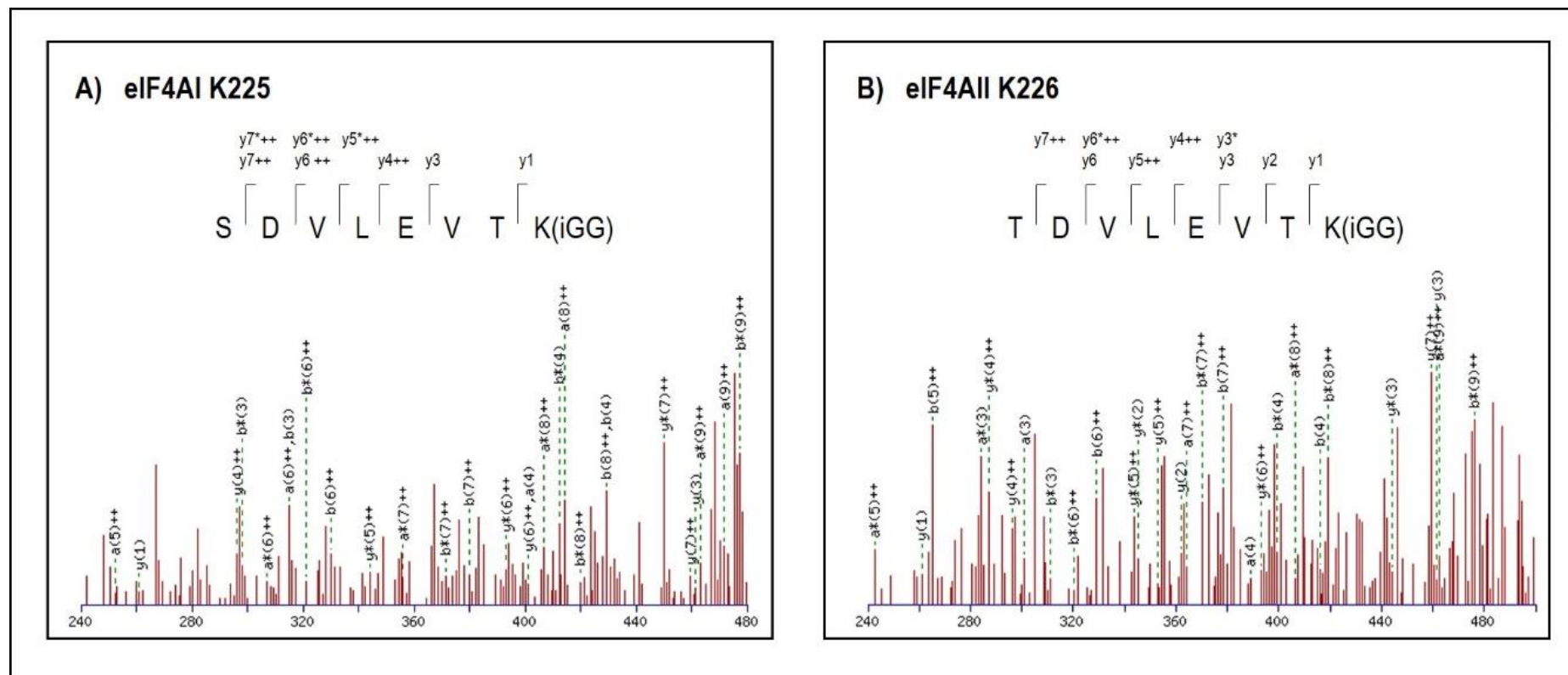


Figure 6.2 Mass spectra of human eIF4AI and eIF4AII. Both proteins are modified by diglycine (GG) motif with high confidence. Mass spectrum of **A)** eIF4AI and **B)** eIF4AII peptides modified by GG motif at K225 and K226, respectively.

eIF4AI	210	VLLSATMP SDVLEVT K KFMRDPIRILVKKE	239
eIF4AII	211	VLLSATMP TDVLEVT K KFMRDPIRILVKKE	240
*****:*****			

Figure 6.3 Alignment of sequences of eIF4AI and eIF4AII. The SUMOylation sites of eIF4AI and eIF4AII indicated in red are K225 and K226, respectively.

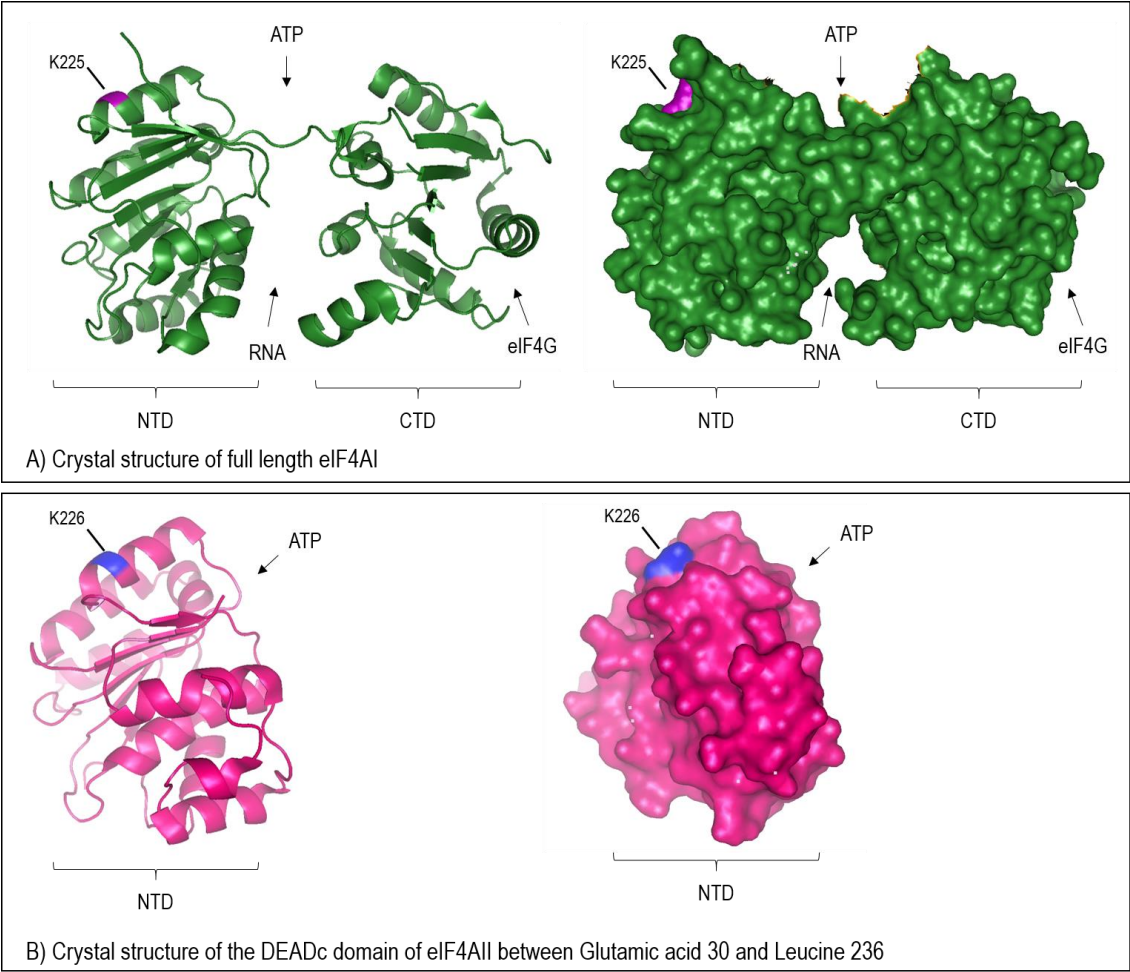


Figure 6.4 Crystal structures of full length eIF4AI and N-terminal eIF4AII. Figures A) eIF4AI (PDB ID 2ZU6) and B) eIF4AII (PDB ID 3BOR) show K225 of eIF4AI and K226 of eIF4AII located on the outer surface of their molecules. In addition, binding surfaces of ATP, RNA and eIF4G are indicated (Oberer *et al.*, 2005). NTD and CTD are N-terminal and C-terminal domains of eIF4A proteins.

6.3 Human eIF4A is modified by both SUMO1 and SUMO2 *in vivo*

To determine whether human eIF4A is modified by SUMO1 or SUMO2 *in vivo*, 6xHis-tagged SUMO1 and SUMO2 were affinity purified using Ni²⁺ agarose beads from HeLa cells, which stably express either 6xHis-tagged SUMO1 (S1) or 6xHis-tagged SUMO2 (S2) (a kind gift from Prof Ron Hay, University of Dundee). Non-transfected HeLa cells (NT) were used as a negative control. Western analysis was carried out using anti-eIF4A antisera, which recognises both eIF4AI and eIF4AII (a gift from Prof. Simon Morley). Figure 6.5 indicates that eIF4A was recovered from cell extracts that contain His-tagged SUMO1 (lane 2) and to a lesser extent from extract containing His-tagged SUMO2 (lane 3). The sizes are consistent with mono- and poly-SUMOylated eIF4A. In contrast, eIF4A was not isolated from normal HeLa cell extracts (lane 1). This indicates that *in vivo* human eIF4A is modified by both SUMO1 and possibly also SUMO2.

6.4 The levels of eIF4A modified by SUMO1 and SUMO2 are altered in response to different stress conditions

HeLa cells containing either His-SUMO1 or His-SUMO2 grown to 60–80% confluence in T75 flasks, were treated with either 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). His-tagged SUMO1 and SUMO2 were affinity purified from cells extracts using Ni²⁺ agarose beads under denaturing conditions as described in section 2.5.19, and compared to the negative control which was HeLa cells untreated (UT) and treated with the same stress conditions (AR, IR). Whole cell extracts (WCE) and affinity purify (PD) samples were loaded onto 10% acrylamide gels. Western blots were probed with anti-eIF4A antisera. Figure 6.6 confirms that eIF4A is SUMOylated by SUMO1 and SUMO2 in untreated cells. Additionally, the eIF4A species in the PD blot represents a poly-SUMOylated form similar in size to the smallest band of the poly-SUMO-eIF4A shown in Figure 6.5. Figure 6.6 also shows that the levels of eIF4A modified by SUMO1 are increased in response to sodium arsenite and ionising radiation. The levels of eIF4A modified by SUMO2 are also slightly increased in response to ionising radiation.

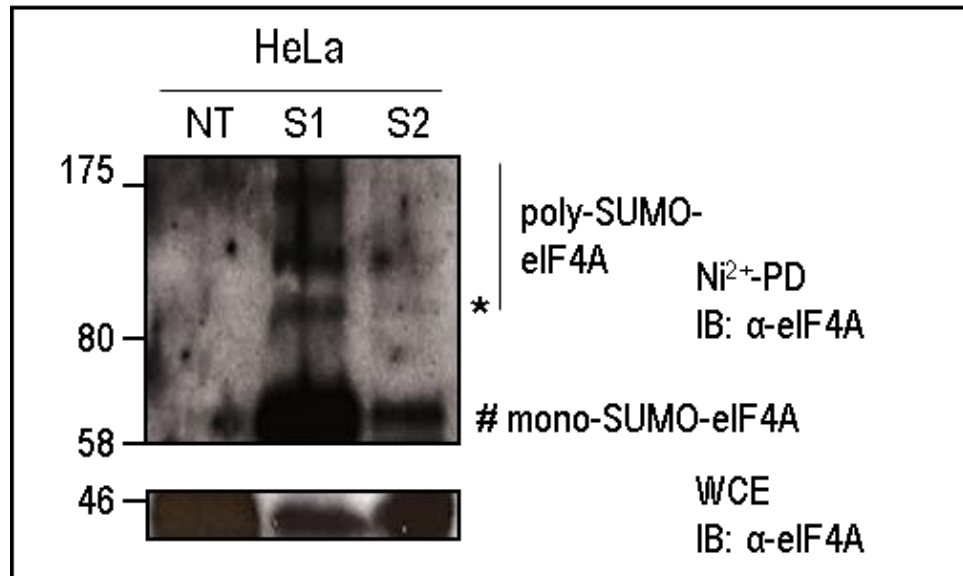


Figure 6.5 Analysis of SUMOylation of eIF4A using Ni²⁺ affinity purification. Whole cell extracts (WCE) and affinity purified samples (PD) from non-transfected HeLa cells (NT) and HeLa cells stably transfected with His-tagged SUMO1 (S1) or SUMO2 (S2) were loaded on 10% gels. Western blots were probed with anti-eIF4A antisera. # represents a mono-SUMOylated eIF4A and * represents the smallest species of poly-SUMOylated eIF4A.

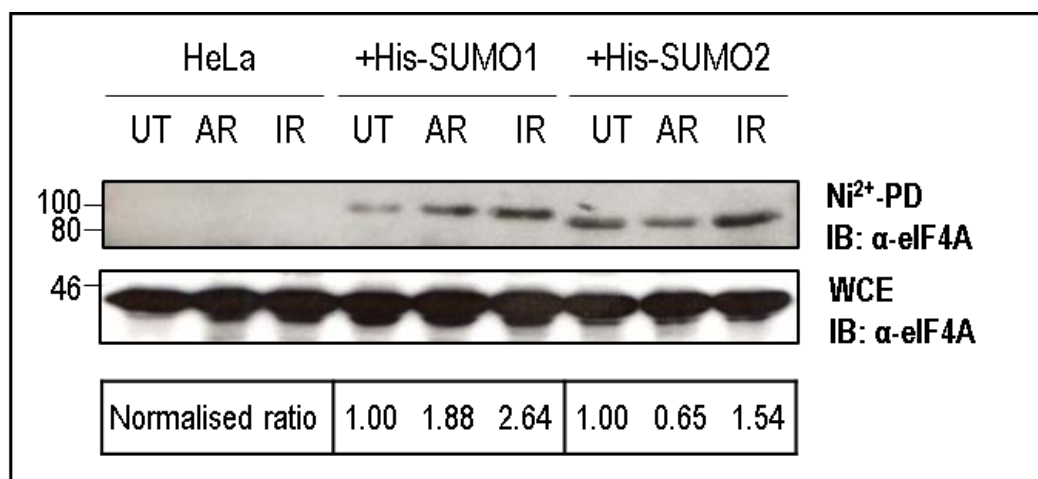


Figure 6.6 Analysis of eIF4A SUMOylation in response to different stresses. Whole cell extracts (WCE) and Ni²⁺ pulled-downs (PD) from wild type HeLa cells and HeLa cells stably transfected with His-tagged SUMO1 or SUMO2 were loaded onto 10% gels. Western blots were probed with anti-eIF4A antisera. Normalised ratio was calculated from the SUMOylated ratio of each stress condition (UT, AR and IR) divided by the SUMOylated ratio of non-treatment (UT). The SUMOylated ratio is the individual intensity of PD result which is divided by the intensity of its WCE result.

6.5 Human eIF4AII is SUMOylated *in vivo*

As anti-eIF4A antisera used in the previous experiment can recognise both eIF4AI and eIF4AII, it was of interest to investigate the modification of eIF4AI and eIF4AII individually by both SUMO1 and SUMO2. However, as only anti-eIF4AII antibody was immediately available in our lab, we focused on the SUMOylation of eIF4AII *in vivo*. Affinity purification using Ni²⁺ agarose beads was undertaken as in section 6.3 but with anti-eIF4AII antibody instead for western blotting. Figure 6.7 indicates that eIF4AII is isolated from cell extracts expressing His-tagged SUMO1 (lane 2) and SUMO2 (lane 3) in PD blot (indicated in arrows), but not from cell extracts of non-transfected HeLa cells (lane 1). This confirms that human eIF4AII is modified by both SUMO1 and SUMO2 *in vivo*. Specifically, eIF4AII is modified by a single SUMO1, while it is modified by SUMO2 as a mono-SUMO2-eIF4AII and poly-SUMOylated eIF4AII.

The size of the species purified with His-SUMO1 is consistent with the size expected for mono-SUMOylated eIF4AII (SUMO1 is unable to form SUMO chains). This size of the mono-SUMOylated eIF4AII (shown as *) may relate to the size of mono-SUMOylated-eIF4A observed in Figure 6.5. In contrast, the species observed following purification of His-SUMO2 likely represent poly-SUMOylated eIF4AII (shown as **). These species correlate with the species between 80 and 175 kDa in the S2 lane in Figure 6.5.

Unfortunately, this figure also shows that the anti-eIF4AII antibody can recognise an 80 kDa non-specific band (indicated as ≠ NS), being present on both blots. This suggests that this antibody may not be good enough for further use e.g. localisation of eIF4AII in cells.

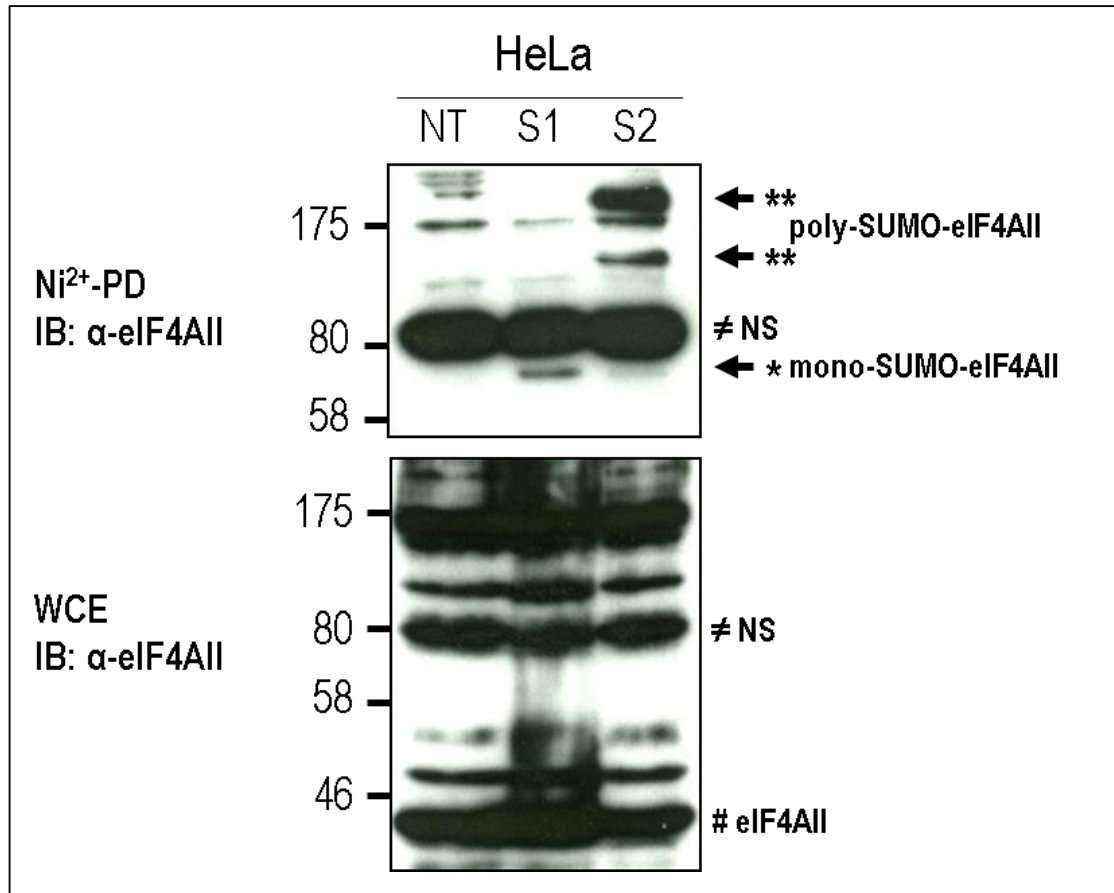


Figure 6.7 Analysis of SUMOylation of eIF4AII using Ni²⁺ affinity purification. Whole cell extracts (WCE) and affinity purified samples (PD) from non-transfected HeLa cells (NT) and HeLa cells stably transfected with His-tagged SUMO1 (S1) or SUMO2 (S2) were loaded on 10% gels. Western blots were probed with anti-eIF4AII antisera. # and ← represent unmodified and modified forms of eIF4AII, respectively, * and ** represent mono-SUMOylated and poly-SUMOylated forms of eIF4AII, respectively. ≠ shows the non-specific protein recognised by the anti-eIF4AII antibody.

6.6 Determining the conditions to knock down eIF4AII using siRNA and for expression of Flag-myc-tagged eIF4AII in HeLa cells

To confirm that lysine 226 (K226) of eIF4AII is the residue that is SUMOylated *in vivo*, it is first necessary to knock down expression of endogenous eIF4AII using siRNA (Meijer *et al.*, 2013). The siRNA-resistant wild type and unSUMOylatable versions of sequences encoding eIF4AII can then be re-introduced into cells for further analysis. Thus it was necessary to establish conditions for knocking down eIF4AII expression.

6.6.1 The condition of knocking down expression of endogenous eIF4AII using siRNA

4×10^5 HeLa cells in 2 ml were plated on a 6-well plate. 5 μ l HiPerFect transfection reagent (Qiagen) was added into 50 μ l Opti-MEM medium, followed by addition of siRNA eIF4AII. The mixtures of eIF4AII siRNA were added to each well to a final concentration of 10, 20 and 30 nM. Cells were incubated at 37°C for 48 hours. Whole cell extracts (WCE) from each sample were loaded onto a gel and compared to WCE from non-siRNA transfected cells (0 nM). A western blot was probed with anti-eIF4AII and anti-tubulin antisera (Figure 6.8). The result shows that using 30 nM siRNA is the optimal concentration for knocking down expression of endogenous eIF4AII, in that it reduces eIF4AII expression approximately three fold.

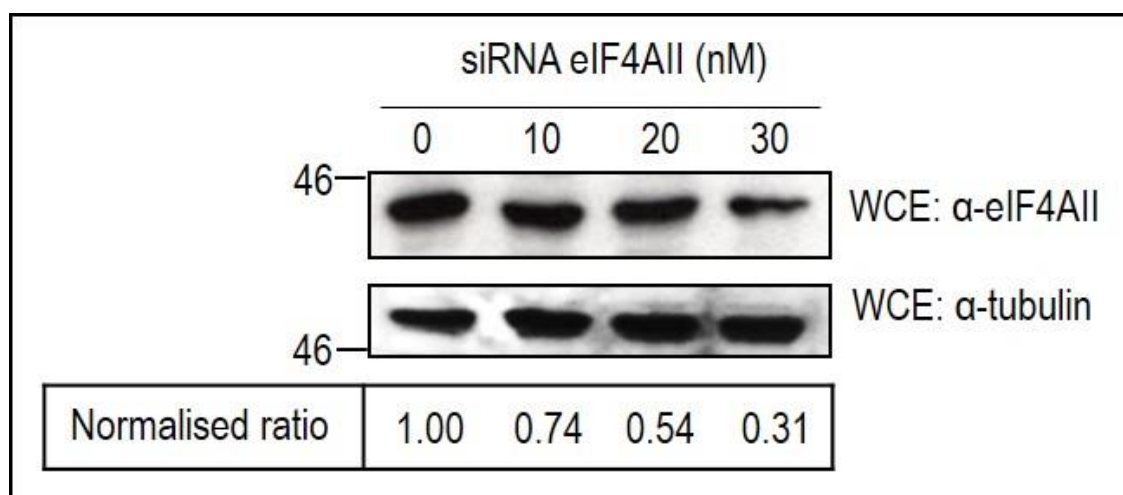


Figure 6.8 Optimisation of siRNA concentration to knock down the expression levels of eIF4AII. The blots were probed with anti-eIF4AII and anti-tubulin antisera. Normalised ratio was calculated from the intensity ratio of eIF4AII to tubulin at each siRNA concentration divided by the intensity ratio of eIF4AII to tubulin at 0 nM siRNA.

6.6.2 Identification of optimal ratio of transfection reagent to DNA plasmid

Plasmid pCMV6 containing eIF4AII (Flag-myc-tagged eIF4AII) was used for expression in HeLa cells (a gift from Prof M Bushell). 4×10^5 HeLa cells in 2 ml were incubated with 30 nM siRNA for 37°C for 48 hours. Plasmid DNA containing Flag-myc-eIF4AII was transfected into the eIF4AII knocked-down cells using the ratio of transfection reagent (FuGENE® HD) to DNA at 3:1 and 3:2. Transfected cells were incubated for a further 24 hours. Whole cell extracts (WCE) were loaded onto a gel, and compared to non-siRNA transfected cells (mock siRNA). A western blot was probed with anti-eIF4AII and anti-tubulin antibodies (Figure 6.9). The result shows that Flag-myc-tagged eIF4AII is expressed well using the transfection reagent to DNA ratio of both 3:1 and 3:2. However it may be better to use a ratio of 3:1 because this results in a similar level of expression as endogenous eIF4AII.

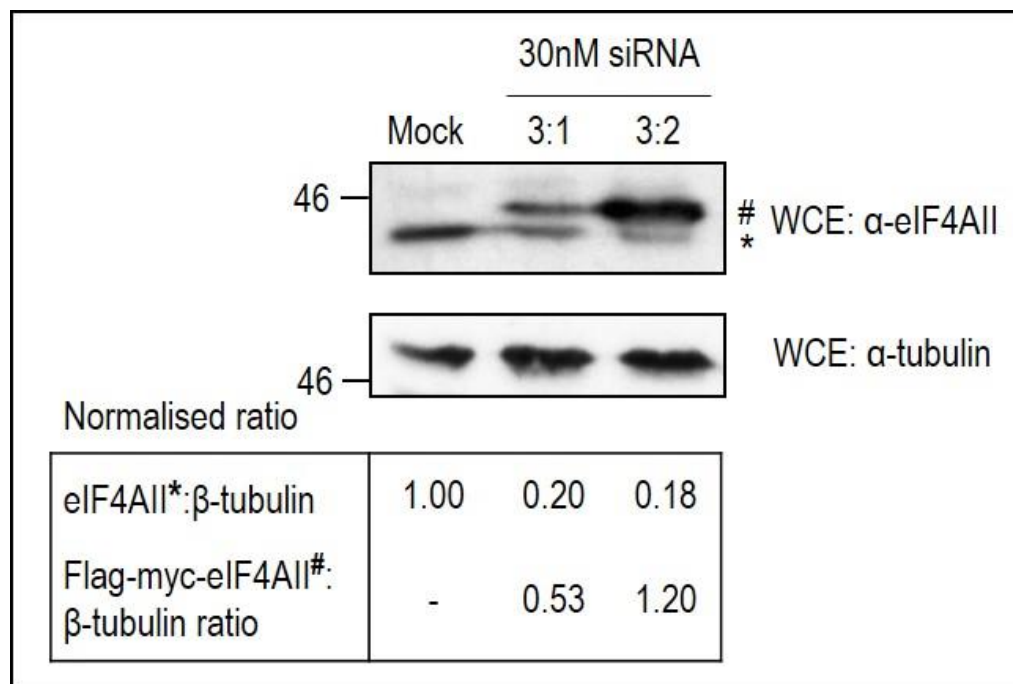


Figure 6.9 Western analysis of optimization of transfection reagent to eIF4AII DNA plasmid. HeLa cells transfected with mock siRNA and 30 nM eIF4AII siRNA. eIF4AII-knocked down cells were then transfected with plasmids containing Flag-myc-tagged eIF4AII using two different ratios of transfection reagent to plasmid DNA. Whole cell extracts (WCE) from those cells were loaded onto 10% gels. A western blot was probed with anti-eIF4AII and anti-tubulin antibodies. * refers to the endogenous eIF4AII and # refers to the recombinant eIF4AII (Flag-myc tagged).

6.7 Investigating the role of eIF4AII SUMOylation after introducing the unSUMOylatable eIF4AII into eIF4AII-knocked down cells

Having identified the optimum conditions for eIF4AII knockdown and subsequent expression from a plasmid, the aim was to determine the effect of the K226R mutation on SUMOylation of eIF4AII. To address this, it was first necessary to construct a vector containing the unSUMOylatable residues (K226R) of eIF4AII. Plasmid pCMV6 containing C-terminally Flag-myc-tagged eIF4AII-wt (Prof. Martin Bushell) was used for mutagenesis using a pair of mutagenesis primers; forward primer 5' GTGTTGGAAGT GACCAGAAAATTCATGAGAGATC 3' and reverse primer 5' GATCTCTCATGAA TTTTCTGGTCACTTCCAACAC 3'. The resulting plasmid was sequenced to ensure that it contained the correct sequence (data not shown).

6.7.1 Determination of whether K226 in eIF4AII is important for the modification by SUMO1

To address this, 2.0×10^6 HeLa cells transfected with His-tagged SUMO1 (HeLa S1 cells) (Prof. Ron Hay) were incubated with 30 nM siRNA in a 10-cm dish for 37°C for 48 hours. 12 µg plasmid DNA containing Flag-myc-eIF4AII (either wild-type or mutant version) was transfected into the eIF4AII knocked-down cells using the ratio of transfection reagent to DNA at 3:1. Transfected cells were incubated for a further 24 hours. Cells were harvested for affinity purification using Ni²⁺ agarose beads (described in 2.5.19). Unfortunately, the nonspecific 80 kDa species observed in the PD blot, affected visualisation of SUMOylated eIF4AII. The result from affinity purification (Figure 6.9) shows that, in the mock control lane (positive control, lane 1 in PD), a SUMO1 modified form of eIF4AII is visible. In addition, in the eIF4AII-knocked down lane (negative control, lane 2 in PD), there is no equivalent species. In the cells where wild type and mutant eIF4AII have been reintroduced, no modified forms are detected. The WCE blot indicates that both recombinant proteins are being expressed under siRNA-mediated eIF4AII repression as the Flag-myc-tagged proteins are present in higher molecular weight than the originally endogenous eIF4AII (untagged eIF4AII). The higher molecular weight of the eIF4AII due to the tag would result in SUMOylated species migrating at a similar position to the non-specific 80 kDa species, thereby interfering with the visualisation of the SUMOylated Flag-myc-eIF4AII. For this reason, an investigation of the SUMO2 modification of eIF4AII was initiated, since it is shown

in Figure 6.7 that there were three higher molecular weight bands of eIF4AII modified by SUMO2.

6.7.2 Determination of whether K226 in eIF4AII is important for the modification by SUMO2

HeLa cells stably transfected with His-tagged SUMO2 (Prof. Ron Hay) were used in order to investigate whether K226 on eIF4AII is important for modification by SUMO2. Unfortunately, eIF4AII-knocked down cells transfected with plasmid DNA containing the unSUMOylatable version of eIF4AII did not grow well, unlike those cells containing eIF4AII-wt, which grew well (Figure 6.11). This implies that K226 in eIF4AII may be important and possibly has a role on cell growth. Because the cells containing eIF4AII-K226R mutation did not grow sufficiently, it was not possible to obtain enough cells for further use in affinity purification using Ni^{2+} agarose beads.

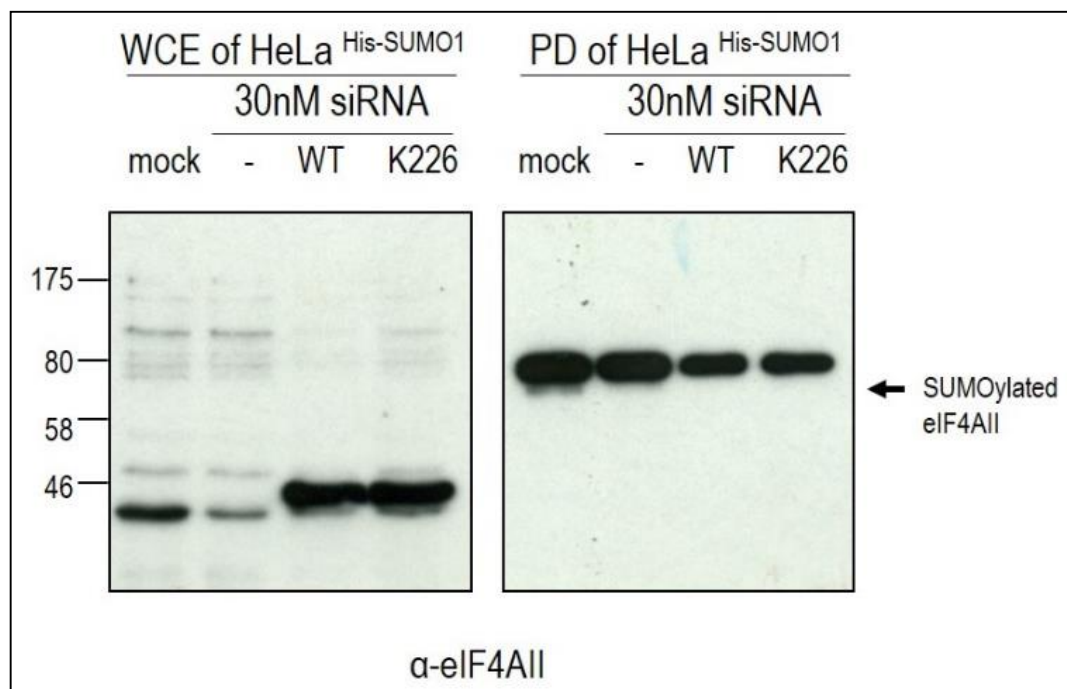


Figure 6.10 Western blotting of Ni^{2+} affinity purification of eIF4AII. The endogenous eIF4AII of HeLa cells stably transfected with His-tagged SUMO1 were knocked down using either non-specific siRNA (mock) or 30 nM siRNA eIF4AII for 48 hours. The eIF4AII-reduced cells were then re-introduced with either wild-type or mutant versions of Flag-myc-eIF4AII. Whole cell extraction (WCE) and Ni^{2+} affinity purification (PD) samples were analysed on 10% PAGE. Blots were probed with anti-eIF4AII antibody.

HeLa^{SUMO2} + siRNA eIF4AII

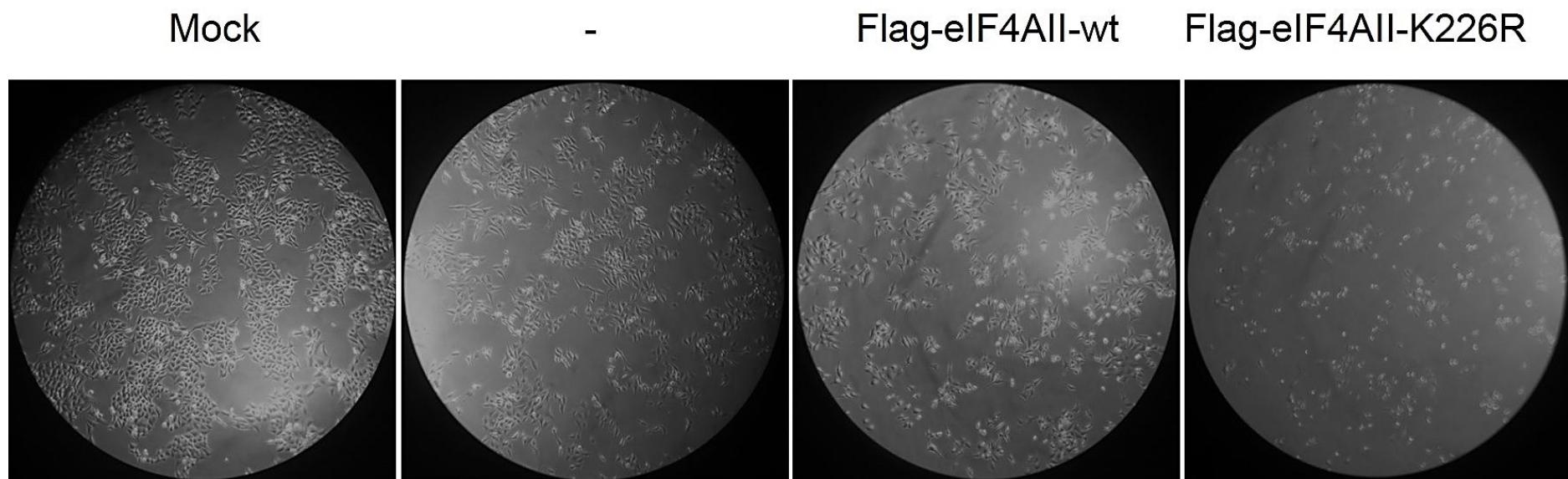


Figure 6.11 Showing cell growth after re-introducing recombinant eIF4AII into the eIF4AII-knocked down HeLa cells containing His-SUMO2. Expression of the endogenous eIF4AII of HeLa cells stably transfected with His-tagged SUMO2 was knocked down using either non-specific siRNA (mock) or 30 nM siRNA eIF4AII for 48 hours. Wild-type (wt) or mutant versions (K226R) of Flag-myc-eIF4AII was then re-introduced into the eIF4A-knocked-down cells for 24 hours. This figure shows that the eIF4AII-knocked down cells containing eIF4AII K226R mutation did not grow well (the rightmost photo). From visual inspection, their number and ability to attach to flasks are significantly lower than others.

6.8 The localisation of SUMO1 and eIF4A in cells exposed to different stress conditions

HeLa cells grown to 60–80% confluence were treated with and without either 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). For microscopy, untreated (UT) and treated cells (AR and IR) cells were incubated with two primary antibodies, namely rabbit anti-eIF4A and mouse anti-SUMO1. To observe the colocalisation of eIF4A and SUMO1 under a fluorescence microscope, FITC anti-mouse IgG and Cy3 anti-rabbit IgG were used against the specific primary antibodies as described in section 2.4.8.

The immunofluorescence result (Figure 6.12) shows that, as was observed with eIF4G, eIF4A (red) is mainly present in the cytoplasm whereas SUMO1 (green) is mostly in the nucleus although there is a small amount in the cytoplasm. In untreated cells there is a low level of colocalisation of eIF4A with SUMO1 in both the nucleus and the cytoplasm (Figure 6.13). In arsenite-treated cells most of the eIF4A is present in stress granules in the cytoplasm although there is still some present in the nucleus. Colocalisation of these proteins is found at the many of the stress granules, particularly at the edges of the granules (Figure 6.14). In IR-treated cells, SUMO1 is present in foci in the nucleus. eIF4A is still mainly in the cytoplasm, but unlike the situation with AR is not present in the stress granules. In these cells, a small amount of eIF4A colocalises with SUMO1 in the nucleus (Figure 6.15). Interestingly, the colocalisation of eIF4A and SUMO1 is increased in HeLa cells exposed to both AR and IR, compared to that in the untreated cells.

These results with eIF4A are similar to those of eIF4G and eIF4E. For example, in untreated cells there is a low level of SUMO1 colocalisation with either eIF4G or eIF4E in both the nucleus and the cytoplasm. In arsenite-treated cells most of eIF4A/eIF4E/eIF4G is observed in the cytoplasmic stress granules. In addition the colocalisation of these three proteins with SUMO1 is found at the many of the edge's stress granules. In IR-treated cells, a small amount of eIF4A, eIF4E and eIF4G colocalises with SUMO1 in the nucleus.

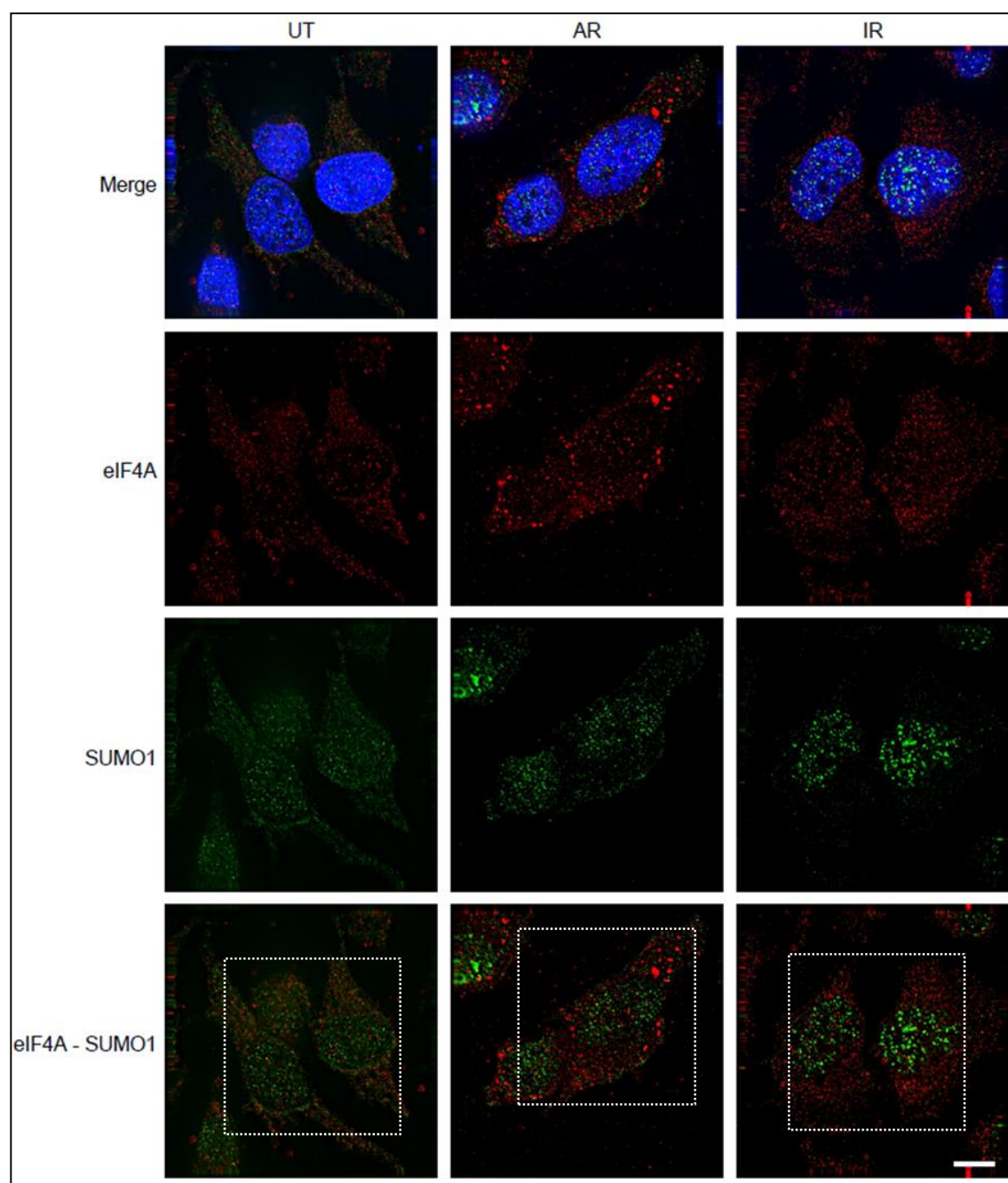


Figure 6.12 Immunofluorescence studies indicating the colocalisation of eIF4A and SUMO1. HeLa cells were untreated (UT) and treated with 1 mM sodium arsenite (AR) or 3 Gy ionising radiation (IR). Blue, red and green colours represent the DAPI-stained DNA, eIF4A and SUMO1, respectively. The scale bar is 10 μ m. The white boxes are shown in the expanded versions as Figure 6.13–6.15.

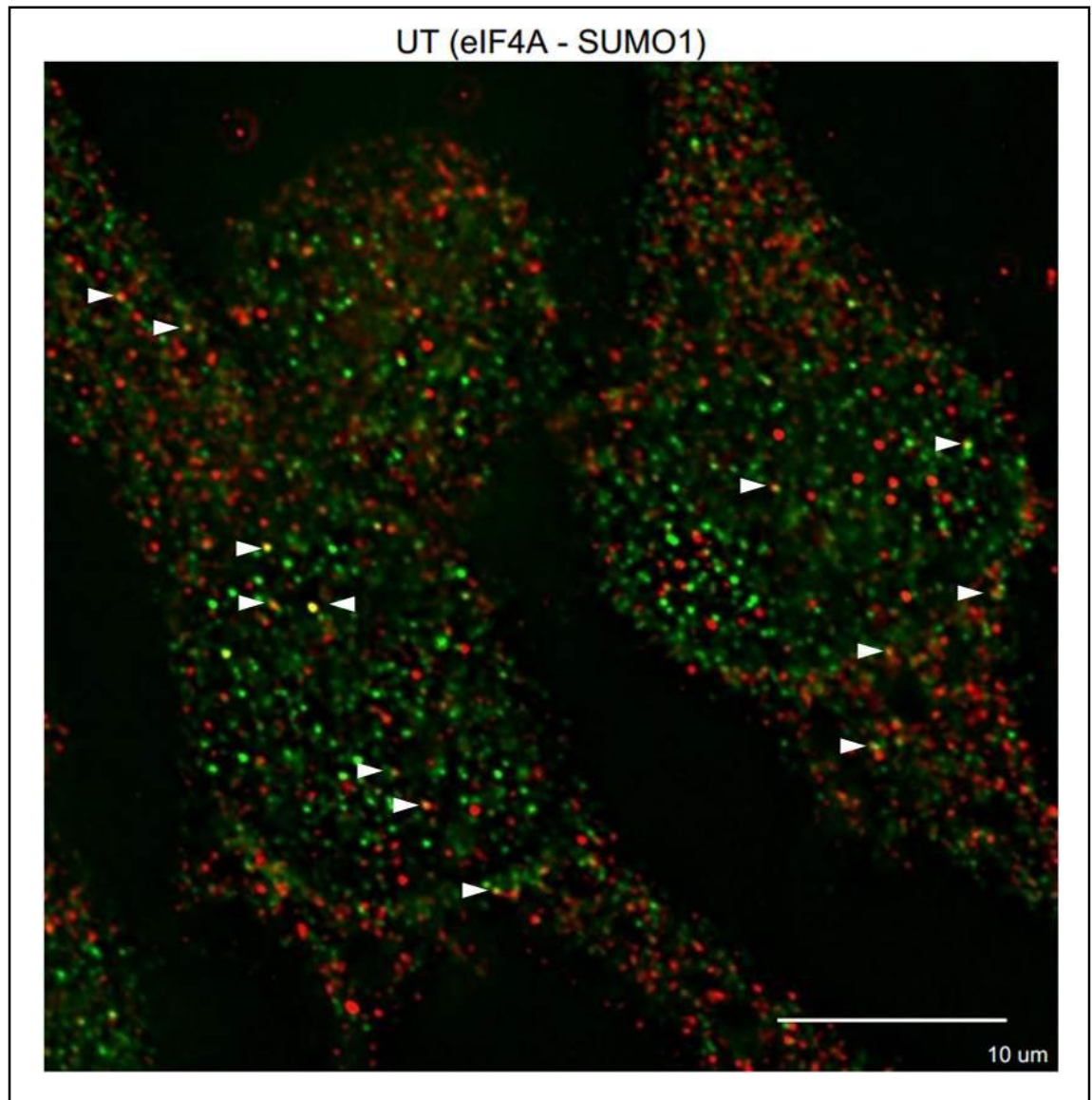


Figure 6.13 Expanded version from Figure 6.12. Immunofluorescence studies of untreated HeLa cells. Red and green colours represent eIF4A and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4A and SUMO1.

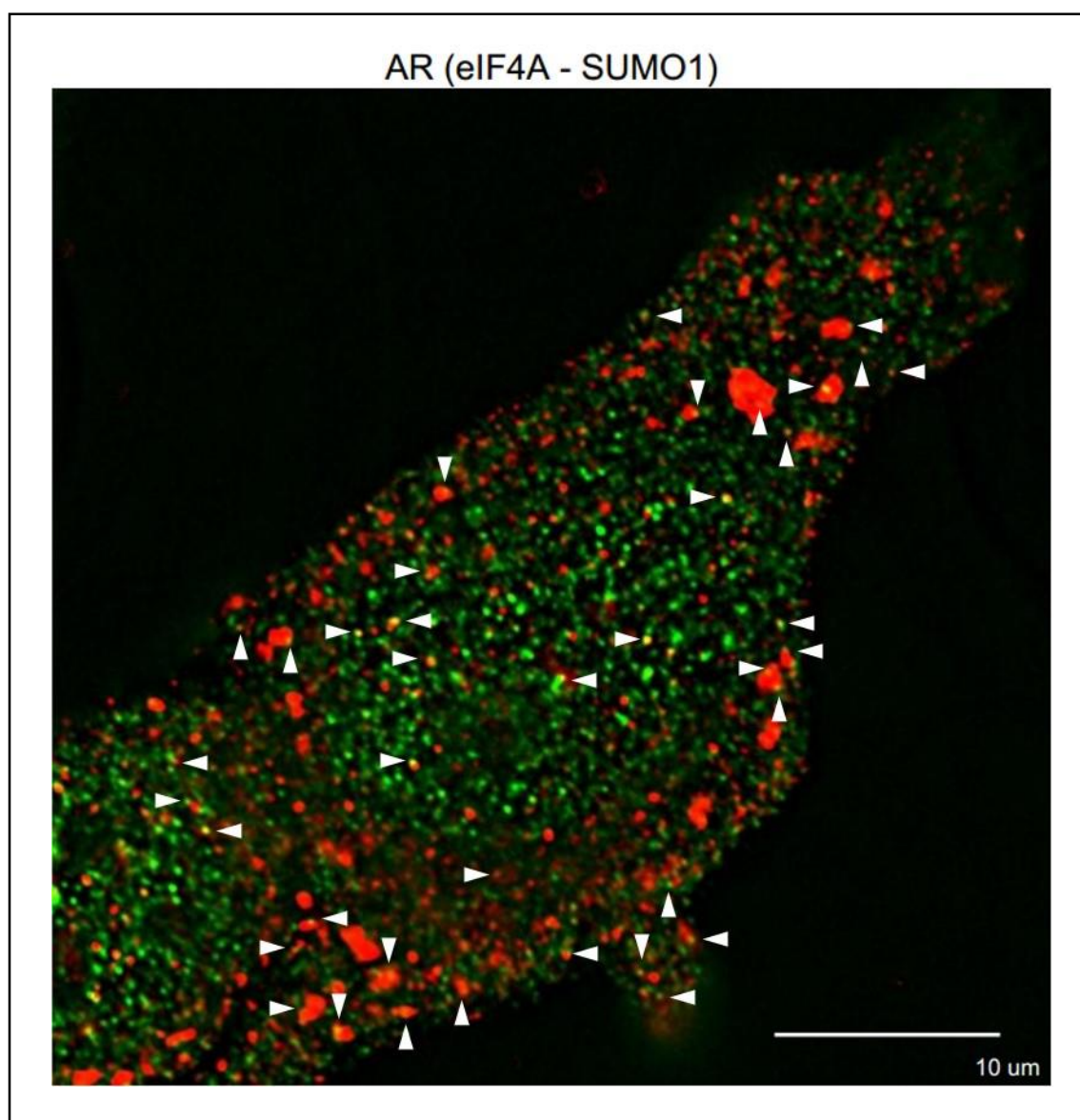


Figure 6.14 Expanded version from Figure 6.12. Immunofluorescence studies of arsenite-treated HeLa cells. Red and green colours represent eIF4A and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4A and SUMO1.

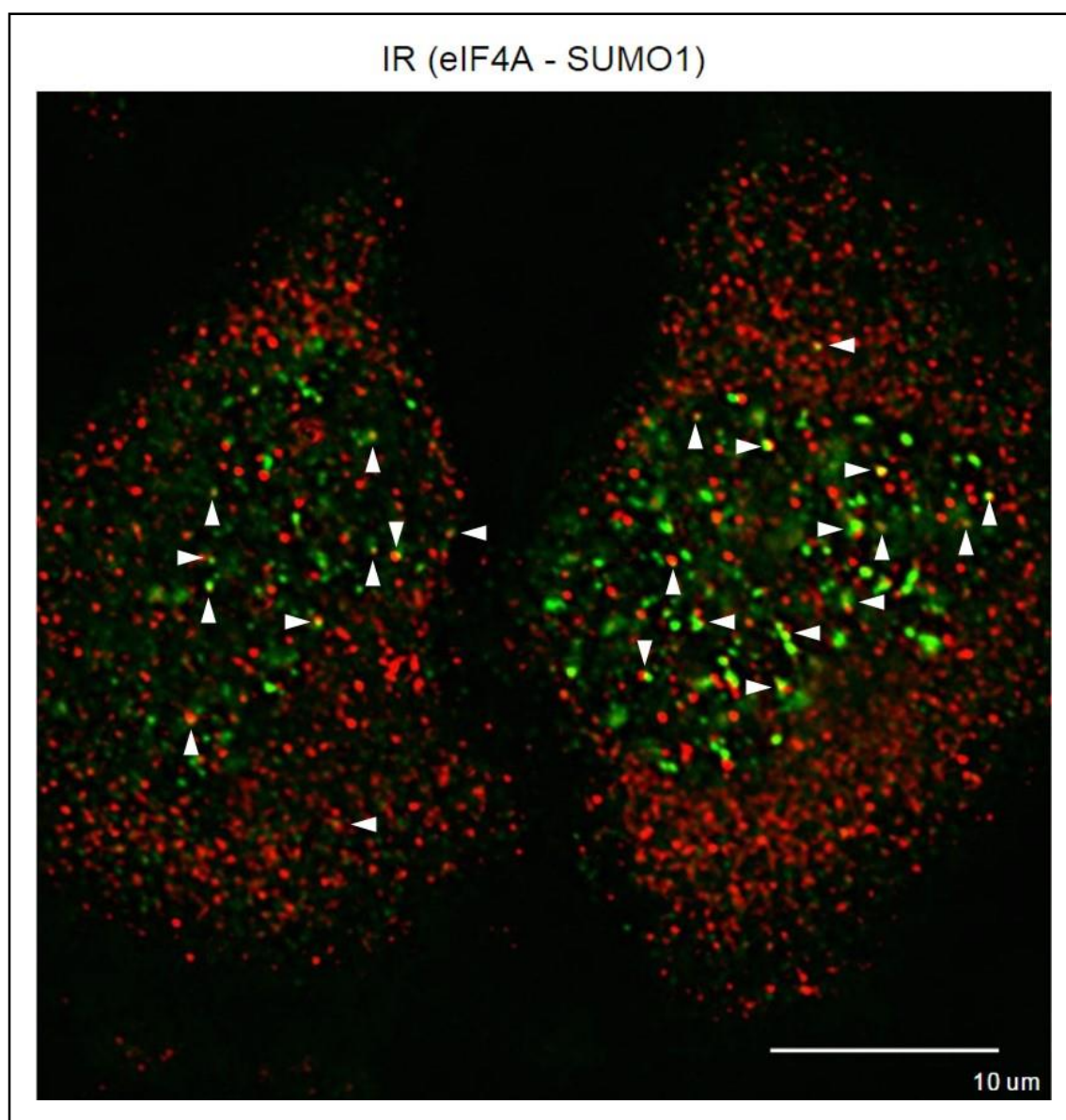


Figure 6.15 Expanded version from Figure 6.12. Immunofluorescence studies of **IR-treated HeLa cells**. Red and green colours represent eIF4A and SUMO1, respectively. The white arrows show sites of colocalisation of eIF4A and SUMO1.

6.9 Discussion

Knowing that human eIF4E and eIF4G are modified by SUMO (Xu *et al.*, 2010; Watts *et al.*, 2014) we were prompted to investigate whether eIF4A is also SUMOylated since eIF4E, eIF4G and eIF4A are components of the eIF4F complex. Even though there are three eIF4A isoforms characterised in human, only eIF4AI and eIF4AII are believed to be involved in translation (Andreou and Klostermeier, 2013). The possibility of SUMOylation of eIF4A is consistent with proteomic screens, indicating that human eIF4AI (Blomster *et al.*, 2009; Bruderer *et al.*, 2011) and eIF4AII (Matafora *et al.*, 2009), but not eIF4AIII, have been identified to be SUMO targets. Thus we began our analysis of SUMOylation of eIF4AI and eIF4AII *in vitro* (Figure 6.1) using our *in vitro* assay (Ho *et al.*, 2001). We found that eIF4AI and eIF4AII are both modified by SUMO *in vitro* (data not shown) on K225 and K226, respectively, using mass spectrometry (Figure 6.2). It is interesting that these two lysine residues are conserved in only eIF4AI and eIF4AII (Figure 6.3), but not in eIF4AIII. These two lysine residues are located on the outer surface of the molecules, consistent with them being available for SUMOylation. It is interesting that the SUMOylation sites of eIF4AI and eIF4AII are very close to the ATP binding pocket (Figure 6.4), suggesting that SUMOylation of eIF4A proteins may have a role in the interaction of eIF4A and ATP molecules.

Next, it was necessary to confirm that human eIF4A proteins are SUMO modified *in vivo*. Initial observations indicated that eIF4A is modified by SUMO1 and possibly also SUMO2 *in vivo* (Figure 6.5). When this was repeated using cells exposed to arsenite and ionising radiation, it was confirmed that SUMO2 modifies eIF4A in untreated cells (Figure 6.6). Notably, the levels of SUMO1-modified eIF4A are increased in response to arsenite and IR, while the levels of eIF4A modified by SUMO2 are also slightly increased in response to IR. This indicates that, in response to arsenite, SUMOylation of eIF4A may be involved in the process of stress granule formation.

Since anti-eIF4A antisera used here can recognise both eIF4AI and eIF4AII, it was of interest to investigate which isoforms of eIF4A proteins, eIF4AI and/or eIF4AII, are SUMOylated. One of the potential isoforms is eIF4AII, and this was chosen for further study since suitable antibodies against eIF4AII were available in our lab. Affinity purification of His-SUMO confirmed that human eIF4AII is modified by both SUMO1 and SUMO2 *in vivo* (Figure 6.7).

Here, studies were initiated to investigate the role of SUMOylation of eIF4AII. This began with determining the optimal conditions of the siRNA eIF4AII concentration and the ratio of transfection reagent to DNA plasmid. The optimal concentration of siRNA eIF4AII was at 30 nM using HiPerFect transfection reagent, showing that ~70% of endogenous eIF4AII was repressed (Figure 6.8). This is consistent with a recent study where 30nM siRNA eIF4AII was used to knock down the expression of eIF4AII using Dharmafect1 (Meijer *et al.*, 2013). For determining the ratio of transfection reagent (Fugene HD) to DNA plasmid, pCMV6 plasmid containing Flag-myc-eIF4AII was used in this study (provided by Prof. Martin Bushell). The recombinant eIF4AII expressed was tolerant to the siRNA eIF4AII, resulting in rescue of the endogenous levels of eIF4AII that had been repressed (Meijer *et al.*, 2013). Flag-myc-tagged eIF4AII was expressed at good levels using the transfection reagent to DNA ratio of 3:1 and 3:2 (Figure 6.9). However it may be better to use a ratio of 3:1 since using this ratio the expression of the recombinant eIF4AII is similar to that of the endogenous eIF4AII.

To investigate the role of K226 on eIF4AII to be SUMOylated, the siRNA-resistant Flag-myc eIF4AII was mutated at lysine 226 to arginine, then re-introduced into the siRNA-mediated eIF4AII-repressed cells containing stably transfected His-SUMO1. His-SUMO1 pulled down under denaturing conditions using Ni²⁺ agarose beads. Unfortunately, the nonspecific 80 kDa bands were recognised by the anti-eIF4AII antibody, affecting the visualisation of the SUMOylated form of recombinant eIF4AII (Figure 6.10). This made it impossible to determine whether K226 on eIF4AII was modified by SUMO1 since the recombinant eIF4AII is C-terminally tagged with Flag and *myc* sequences, leading to an overall increase in size of eIF4AII. This problem could be resolved in a few ways. (1) A new anti-eIF4AII antibody could be used. (2) Anti-Flag or anti-myc antibodies could be used instead of eIF4AII. However, this latter approach was tried but was not successful as anti-Flag and anti-myc antibodies also recognised a number of other non-specific bands (data not shown). (3) Untagged-eIF4AII could be the alternative, but it may be difficult to distinguish between the original eIF4AII and the re-introduced eIF4AII.

From the reasons described above, investigating the SUMO2 modification of eIF4AII was thought that it might be easier since it is shown in Figure 6.11 that there were three bands of eIF4AII modified by SUMO2. However, there was another difficulty with this experiment. HeLa cells containing His-SUMO2 (Prof. Ron Hay) were used for

this. The cells transfected with plasmid DNA containing the unSUMOylatable version of eIF4AII were sick while those cells containing eIF4AII-wt grew well. This implies that K226 on eIF4AII may be important and possibly has a role in cell growth. However, the number of the cells containing eIF4AII-K226R was not enough for use in His-SUMO purification, but was enough for colocalisation analysis. For the localisation study, using anti-eIF4AII, anti-SUMO2/3, anti-Flag and anti-myc antibodies was unsuccessful since the antibodies recognised non-specific proteins.

Work therefore focussed on the localisation of eIF4A proteins and SUMO1 using anti-eIF4A and anti-SUMO1 antibodies (Figure 6.12). The result shows that in arsenite-treated cells SUMO1 colocalises with eIF4A in the cytoplasmic stress granules, especially at the edges of the stress granules (Figure 6.14). In contrast, in IR-treated cells (Figure 6.15), it was found that eIF4A colocalises with SUMO1 more in the nucleus, compared to the colocalisation of SUMO1 and eIF4A in untreated cells (Figure 6.13). This is consistent with the result that the SUMOylation levels of eIF4A are increased in response to the genotoxic stress. This suggests that the SUMOylation of eIF4A may have a role involved in nuclear processes.

Chapter 7

Conclusion

To investigate the SUMOylation of proteins, we have used an *in vitro* SUMOylation assay (Ho *et al.*, 2001) and mass spectrometry in order to identify SUMOylated lysine residues on the proteins. In addition, we have analysed SUMOylation of the proteins *in vivo*. However, it is found that there were several limitations to the investigation of the role of SUMOylation of the proteins studied, particularly of *S. pombe* eIF4G, human eIF4G and human eIF4A.

7.1 Limitations encountered during this study

The limitations cover a number of aspects. First, in studying SUMOylation of proteins *in vivo*, it was difficult to identify and isolate SUMO conjugates from cell extracts even though SUMO protease inhibitors e.g. N-ethylmaleimide (NEM) were added to immunoprecipitation lysis buffers, which involved the purification of proteins under native conditions. This led to analysis of SUMOylation of proteins using only Ni²⁺ affinity purification, which was undertaken using denaturing conditions which would prevent the activity of SUMO proteases.

For studying SUMOylation of *S. pombe* proteins, it was first necessary to tag the proteins with epitopes or sequences because specific antibodies against all proteins were not available. Fortunately, *S. pombe* containing eIF4G tagged with HA epitopes has been available (made by Dr. Felicity Watts), so there was no problem on the specific antibody to *S. pombe* eIF4G. However, another difficulty was at the step of cloning the full length *S. pombe* eIF4G cDNA which would be needed for *in vitro* SUMOylation assays in order to identify SUMOylated lysine residues using mass spectrometry. Hashemzadeh-Bonehi *et al.* (2003) have shown that plasmids containing sequences encoding the N-terminal *S. pombe* eIF4G are unable to be tolerated in *E. coli*. The reason for this is unknown, but is possibly due to the presence of a highly repeated sequence within *S. pombe* eIF4G (this is not found in the *S. cerevisiae* or human proteins). Additionally, the highly repeated sequence of *S. pombe* eIF4G contains 16 repeats of a perfect SUMOylation site consensus motif (AKRE), which would be likely to make it difficult to identify the SUMOylation sites, even if the full length eIF4G was able to be expressed in *E. coli*.

Unlike antibodies against *S. pombe* proteins, most specific antibodies to human proteins are commercially available. However, some antibodies used in this study were able to recognise non-specific epitopes or sequences. For example, Figure 6.7 shows that anti-eIF4AII antibody was able to recognise an 80 kDa non-specific protein in both whole cell extract and affinity purification blots. Additionally, in localisation studies, anti-SUMO2/3, anti-Flag and anti-myc antibodies were used to represent SUMO2/3 and Flag-myc-eIF4AII in cells, but they all recognised other non-specific epitopes (data not shown), thus confusing the images.

7.2 Conclusions

Here, this study has shown that *S. pombe* eIF4G and Sla1 are SUMOylated *in vivo*, but eIF3h is not. In addition, the level of SUMOylated eIF4G was increased after treatment with osmotic stress conditions (1 M KCl, a stress granule inducer) compared to the levels in untreated cells. The role of this modification is not known. However, there are two possibilities. The first, is that SUMOylation may be targeting eIF4G for degradation, possibly via the action of a SUMO-targeted ubiquitin ligase (STUbL). The second possibility is that SUMOylation may be targeting eIF4G to stress granules or P-bodies. Further studies conducted here indicated that the eIF4G and Ulp2-containing complexes are unlikely to be polysomes. This would be consistent with SUMOylation targeting eIF4G to stress granules or P-bodies. For further work for studying the role of *S. pombe* eIF4G, it might be worth another attempt to clone full length eIF4G ORF or truncated versions of eIF4G. The expressed protein could be used for *in vitro* SUMOylation assays in order to identify SUMOylated lysine residues using mass spectrometry.

Next, human eIF4G is SUMOylated *in vivo* and *in vitro*. Two SUMOylation sites on human eIF4G, K1386 and K1588, were identified which are not conserved in the fission yeast eIF4G. This is because the *S. pombe* protein does not contain the C-terminal domains present in human eIF4G. SUMO modification on these sites in human eIF4G may affect interactions of eIF4G with eIF4A and Mnk1. As previously described, eIF4A is a DEAD-box protein that is involved in translation initiation. It binds to eIF4G (Sonenberg and Hinnebusch, 2009; Jackson *et al.*, 2010) in order to unwind secondary structure in the 5' UTR of mRNAs as being an ATP-dependent RNA helicase. The activities of eIF4A (both an RNA-dependent ATPases and an ATP-dependent RNA

helicase) are enhanced by its interaction with two domains on eIF4G, locating at the middle and C-terminal domains (Sonenberg and Hinnebusch, 2009; Jackson *et al.*, 2010). Results here suggest that SUMOylation of eIF4G may affect the interaction with eIF4A, possibly leading to the regulation of translation initiation. Mnk1 is a kinase which can bind the C-terminal eIF4G. It has a role in the translational regulation through phosphorylation of eIF4E at Ser209 (Pyronnet *et al.*, 1999; Sonenberg and Hinnebusch, 2009; Jackson *et al.*, 2010). Phosphorylated eIF4E has been shown to be modified by SUMOylation on five lysine residues, leading to the formation of eIF4F complex and specific protein synthesis (Xu *et al.*, 2010). It is possible that the SUMOylation of eIF4G at K1588 may prevent the binding of Mnk1 to eIF4G and reduce phosphorylation of eIF4E. If it is the case, SUMOylation of eIF4E and the formation of eIF4F complex may be interfered, leading to decrease the translation of specific mRNA.

Like human eIF4G, human eIF4A is also SUMOylated *in vivo* and *in vitro*. The SUMOylation sites on human eIF4AI and eIF4AII were K225 and K226, respectively. These sites are highly conserved in both isoforms because these two isoforms share 90-95% sequence identity which is mostly different at the N' terminus. Interestingly, the SUMOylation sites of both eIF4A isoforms are close to the ATP binding pocket. It suggests that SUMOylation of eIF4A may have a role in the interaction of eIF4A and ATP. However, the role of SUMO1 modification of K226 on eIF4AII was further analysed since eIF4AII is thought to involve in a miRNA-mediated translational repression (Meijer *et al.*, 2013). One of our collaboration also has an assay in order to determine the role of SUMOylation of eIF4AII in the context of the miRNA-mediated translational repression. As one of the limitations was described above, the nonspecific 80 kDa bands were recognised by anti-eIF4AII antibody, affecting the visualisation of the SUMO1-conjugated form of recombinant eIF4AII. Thus, investigating the SUMO2 modification of eIF4AII might be easier since there were three bands of eIF4AII modified by SUMO2 (Figure 6.7). However, there was another difficulty since eIF4AII-knocked down HeLa cells containing His-SUMO2 were sick when they contained the unSUMOylatable version of eIF4AII. This implies that K226 on eIF4AII may be important and possibly has a role on cell growth. In addition, the number of the cells containing eIF4AII-K226R was not enough for use in His-SUMO purification. These results suggest that the unSUMOylatable eIF4AII may have a 'dominant negative' effect in cells.

Colocalisation of human eIF4A/eIF4G and SUMO1 was then analysed in cells. It shows that, in arsenite-treated cells, SUMO1 colocalises with eIF4A and eIF4G (including eIF4E) in the cytoplasmic stress granules, especially at the edges of the stress granules. In contrast, in IR-treated cells, it is found that eIF4G/eIF4A colocalises with SUMO1 more in the nucleus, compared to the colocalisation of SUMO1 and eIF4G/eIF4A in untreated cells. This suggests that eIF4G/eIF4A and SUMO1 may have a cellular role in some aspects in response to arsenite and ionising radiation. To address this, further works need to be done.

From the results described above, we conclude that, like human eIF4E, human eIF4G and human eIF4A are modified by SUMO1. We also show that these three proteins in the eIF4F complex are modified by SUMO2.

7.3 Further work

In order to fully determine, the role of SUMOylation of eIF4G, eIF4AI and eIF4AII further work needs to be undertaken. For example, more specific anti-SUMO2/3 may be necessary, in particular, in the study of the localisation of these translation factors with SUMO2/3 in cells. Specific anti-eIF4AI antibody could be used in order to investigate the role of SUMOylation of eIF4AI. Anti-eIF4AII antibody, which is more specific than the antibody here, is also needed in order to determine whether K226 in eIF4AII is important for the modification by SUMO1 and SUMO2. Additionally, it would be very interesting to investigate why eIF4AII-knocked down cells containing the unSUMOylatable version of eIF4AII did not grow well while those cells containing eIF4AII-wt grew well (Figure 6.11).

It may also be interesting to have clones of other cell types which have been stably transfected with either His-SUMO1 or His-SUMO2 e.g. MCF7 and MDA-MB231 cells. Since, in those cell types, the levels of SUMOylation has been altered in response to arsenite and ionising radiation.

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Appendix

A1 SUMO1 modification of endogenous eIF4G observed by immunoprecipitation (IP) technique

HeLa cells were cultured until 70–80% confluence in T-75 flasks. Then, cells were treated either with or without the following stress conditions: (i) heat shock (HS), at 42°C for 15 minutes and re-incubation at 37°C for 30 minutes, (ii) 3 Gy ionising radiation (IR), followed by re-incubation at 37°C for 30 minutes and (iii) 10 μ M MG-231 (MG) proteasome inhibitor for 30 minutes. Cells were harvested and used for immunoprecipitation using anti-eIF4G antisera and protein A agarose beads as previously described in section 2.5.17. The result in figure A1 shows both unmodified (*) and modified (***) bands of eIF4G. It is clear that eIF4G is modified by SUMO1 when expressed at the endogenous levels. It also confirms that eIF4G is not recovered by protein A beads alone in the absence of anti-eIF4G antisera. However, there is no change of SUMOylation level of eIF4G in response to different stress conditions.

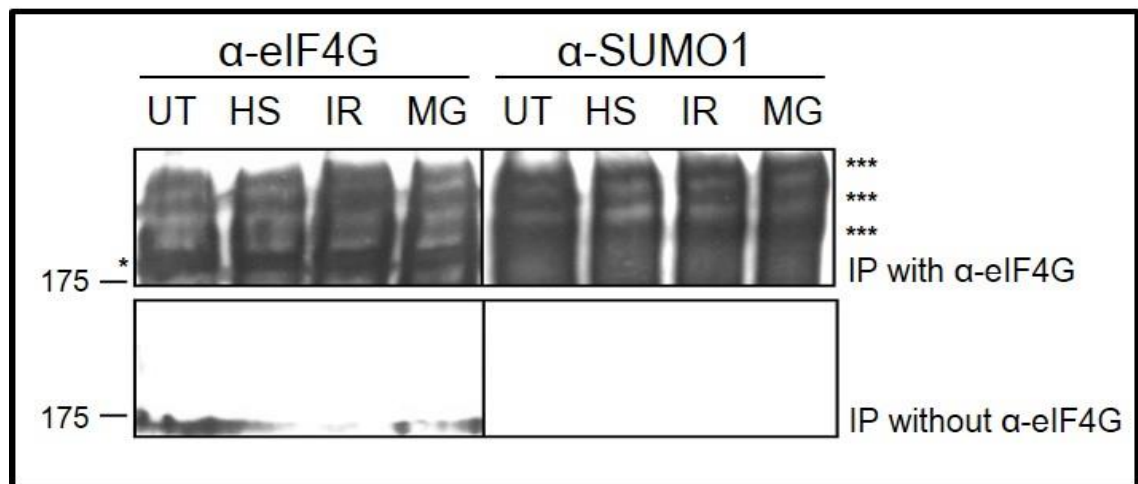


Figure A1 Western blotting of immunoprecipitation (IP) using anti-eIF4G antisera and protein A beads. IP samples were loaded onto 7.5% gels. HeLa cells, untreated (UT) and treated with either heat shock (HS), 5 Gy IR or 10 μ M MG132 (MG), were immunoprecipitated with and without anti-eIF4G antisera. The blots were probed with anti-eIF4G antisera and anti-SUMO1 antibody. (*) indicating unmodified form of eIF4G, *** indicating SUMOylated eIF4G)

A2 Purification of eIF4F complexes using m⁷GTP Sepharose beads

The eIF4F complex which contains several eIF3 subunits was partially purified using m⁷GTP Sepharose beads. Western blotting with anti-eIF3 antisera, anti-SUMO1 and anti-SUMO2/3 antibodies shows that there are numerous SUMOylated species in the preparation. Interestingly, SUMO1 appears to modify the smaller eIF3 subunits in both MRC5 and MCF7 cells, while SUMO2/3 is present in higher molecular weight species. This could represent that small molecular weight proteins are SUMOylated as single by SUMO1. Moreover, SUMO modification of these subunits decreases in response to IR, particularly in MCF7 cells. Interpretation of these results is difficult because eIF4F complexes consist of several factors – eIF4G, eIF4E, eIF4A – so that identification of individual proteins that are SUMOylated is difficult. However, it could be repeated in order to observe SUMOylation of other eIFs e.g. eIF4G, eIF4E and/or eIF4A as specific antisera are available for these proteins. It could also be extended to study SUMOylation of eIFs in response to the stresses as above.

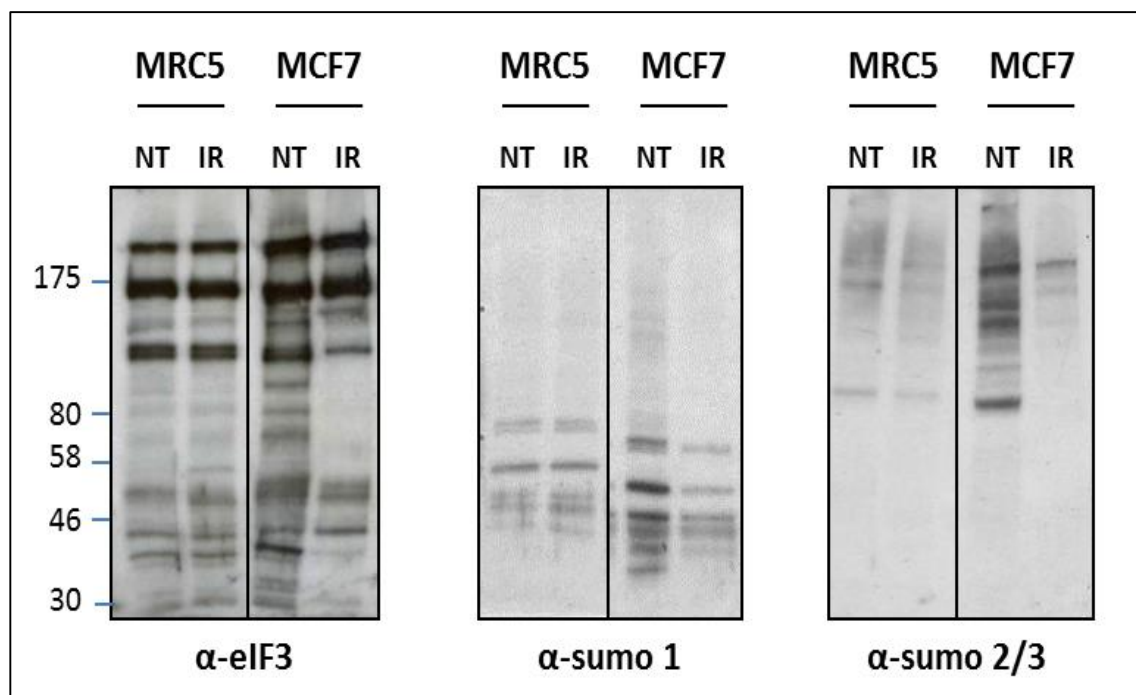


Figure A2 Western analysis of partial purification of eIF4F complexes using m⁷GTP Sepharose. Blots were probed with anti-eIF3 antisera, anti SUMO1 and SUMO2/3 antibodies.

A3 Characterisation of anti-eIF4A and anti-eIF4AII antibodies

This aimed to determine whether the antibodies, eIF4A (in-house antibody made by Prof Simon Morley) and eIF4AII (sc-137147) are able to distinguish between eIF4AI and eIF4AII. The recombinant eIF4A proteins, His-tagged eIF4AI and GST-tagged eIF4AII were expressed in *E. coli* and then purified under denaturing conditions (performed by Robert Baldock, University of Sussex). These samples were loaded on 10% acrylamide gels. Blots were then incubated with either anti-eIF4A or anti-eIF4AII antibodies. Results in Figure A3 show that anti-eIF4A antibody recognises both eIF4AI and eIF4AII isoforms while anti-eIF4AII antibody is able to specifically recognise eIF4AII isoform. This suggests that anti-eIF4AII antibody (sc-137147) is good enough to distinguish the different isoforms of eIF4A effectively.

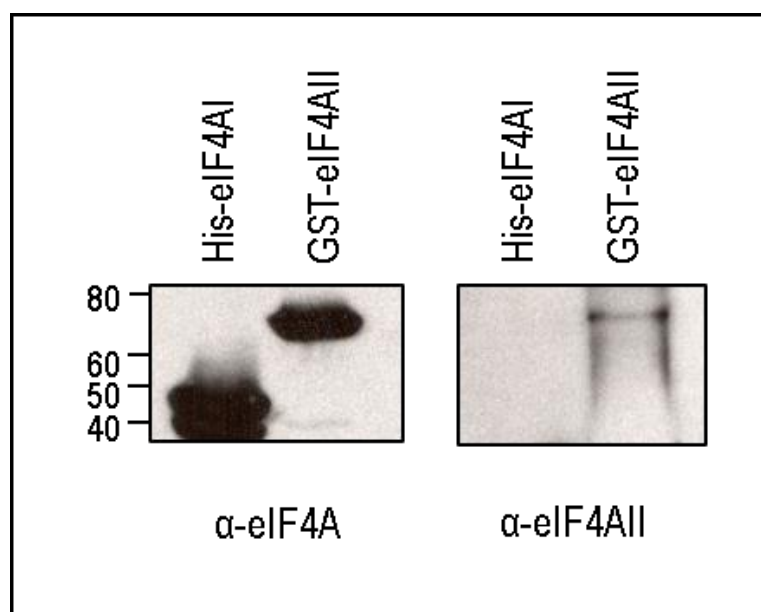


Figure A3 Western analysis for characterisation of anti-eIF4A and anti-eIF4AII antibodies. His-eIF4AI and GFP-eIF4AII were expressed and purified from *E. coli*. This figure indicates that anti-eIF4A antisera can recognise both eIF4AI and eIF4AII isoforms, but anti-eIF4AII antibody can recognise only eIF4AII.

List of Publications

- Jongjitwimol, J.**, Feng, M., Zhou, L., Wilkinson, O., Small, L., Baldock, R., Taylor, D.L., Smith, D., Bowler, L.D., Morley, S.J. & Watts, F.Z. (2014) The *S. pombe* translation initiation factor eIF4G is sumoylated and associates with the SUMO protease Ulp2. *Public Library of Science One*, 9 (5), e94182.
- Watts, F.Z., Baldock, R., **Jongjitwimol, J.** & Morley, S.J. (2014) Weighing up the possibilities: Controlling translation by ubiquitylation and sumoylation. *Translation*, 2 (2), e959366.



The *S. pombe* Translation Initiation Factor eIF4G Is Sumoylated and Associates with the SUMO Protease Ulp2

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Abstract

SUMO is a small post-translational modifier, that is attached to lysine residues in target proteins. It acts by altering protein-protein interactions, protein localisation and protein activity. SUMO chains can also act as substrates for ubiquitination, resulting in proteasome-mediated degradation of the target protein. SUMO is removed from target proteins by one of a number of specific proteases. The processes of sumoylation and desumoylation have well documented roles in DNA metabolism and in the maintenance of chromatin structure. To further analyse the role of this modification, we have purified protein complexes containing the *S. pombe* SUMO protease, Ulp2. These complexes contain proteins required for ribosome biogenesis, RNA stability and protein synthesis. Here we have focussed on two translation initiation factors that we identified as co-purifying with Ulp2, eIF4G and eIF3h. We demonstrate that eIF4G, but not eIF3h, is sumoylated. This modification is increased under conditions that produce cytoplasmic stress granules. Consistent with this we observe partial co-localisation of eIF4G and SUMO in stressed cells. Using HeLa cells, we demonstrate that human eIF4GI is also sumoylated; *in vitro* studies indicate that human eIF4GI is modified on K1368 and K1588, that are located in the C-terminal eIF4A- and Mnk-binding sites respectively.

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Introduction

Sumoylation is a post-translational protein modification that is required for numerous processes within cells, including transcription, chromosome segregation, DNA damage responses, cell signalling and meiosis (reviewed in [1–7]). At the molecular level it functions by altering the surface of target molecules to affect protein-protein interactions e.g. of PCNA (proliferating cell nuclear antigen) and Srs2 (a DNA helicase) [8,9], by altering the intracellular localisation of proteins e.g. of RanGAP [10], or by changing the conformation of target proteins (e.g. in the case of thymine DNA glycosylase [11]). SUMO chains attached to target proteins can also be ubiquitinated and thus result in proteolysis of the target.

SUMO is a small ubiquitin-like modifier that is attached to lysine residues in target proteins. The yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* both have a single gene for

SUMO: *pmt3* and *SMT3*, respectively, while mammals have four, SUMO-1, -2, -3 and -4 (although the role of SUMO-4 is not well defined). SUMO-2 and -3 are 97% identical to each other and about 50% identical to SUMO-1 (reviewed in [1]). SUMO is produced as a precursor protein that needs to be cleaved into the mature form in order to act as a substrate in the sumoylation reaction. Processing of SUMO requires a specific SUMO-protease [12–14], and involves the removal of a small number of amino acids from the C-terminus of precursor SUMO to reveal a Gly-Gly motif. Mature SUMO is then activated by the formation of a thioester bond between the C-terminal glycine residue and a cysteine residue in one subunit of the SUMO activating enzyme (E1). From here SUMO is passed to the SUMO conjugating enzyme (E2), where it again forms a thioester bond with another cysteine residue. SUMO can then be attached to one or more lysine residues in the target protein. In some cases, one of a small

number of SUMO ligases (E3) is required for conjugation. In many cases the lysine is contained within the consensus motif ψ Kx ϵ , where ψ is a hydrophobic amino acid, and x is any amino acid. SUMO can be added to target proteins as a monomer or as poly-SUMO in the form of chains. The removal of SUMO from target proteins or dismantling of SUMO chains occurs via the action of SUMO-specific proteases [14,15].

In *S. cerevisiae* there are two SUMO proteases, Ulp1 and Ulp2, both of which can deconjugate SUMO from target proteins, but which have different target specificities [12]. Only Ulp1 is capable of processing precursor SUMO to the mature form [12,15]. Ulp1 and Ulp2 are differently localised within the cell: Ulp1 is located at nuclear pores, while Ulp2 is located mainly within the nucleus [15]. Mammalian cells have six SUMO-specific proteases (SENPs). These are also differentially localised within cells and have different abilities to cleave precursor SUMO and to deconjugate SUMO from targets e.g. [16,17]. The *S. pombe* Ulp1 protease has been characterised and shown to process SUMO to the mature form, and like *S. cerevisiae* Ulp1, to be located at the nuclear periphery [13]. However, little is known about Ulp2 in this organism.

Translation initiation factors, which play key roles in cell survival and oncogenesis [18–22], can be modified by sumoylation [6,7,23–31]. Protein synthesis is carried out in three stages (initiation, elongation and termination), with the initiation stage of translation generally accepted as a major site of regulation of gene expression in mammalian cells [18–22]. This step in protein synthesis is regulated by a family of proteins, the initiation factors [18,21,22] which interact with each other and the mRNA. These proteins modulate the binding of mRNA to the ribosome, a process facilitated by the assembly of the cap binding protein (eIF4E), a helicase (eIF4A) and a scaffold protein (eIF4G), to form the eIF4F complex (eIF4E/eIF4A/eIF4G). The eIF4G scaffold protein possesses domains that interact with eIF4E, eIF4A, eIF3 and the poly(A) binding protein (PABP) [18,20–22]. The activity of the eIF4F complex is regulated by a family of proteins, the eIF4E binding proteins (4E-BPs). Using a conserved motif, 4E-BPs compete with eIF4G for a common surface on eIF4E and inhibit eIF4F assembly. In mammalian cells, activation of the mechanistic target of rapamycin (mTORC1) leads to the multi-site phosphorylation of 4E-BP1 [18,22,32] preventing 4E-BP1 from binding to eIF4E and thereby allowing formation of the eIF4F initiation complex and ribosomal recruitment of mRNA [18,21,22]. More recently, phosphorylated human eIF4E has been shown to be modified by sumoylation on five lysine residues [33]. Consistent with a role in modulating protein-protein interactions [34], sumoylation did not interfere with mRNA recognition but enhanced eIF4F complex level assembly on the mRNA cap, promoting the expression of ornithine decarboxylase, c-myc and Bcl-2, thereby driving the anti-apoptotic and oncogenic activity of eIF4E [33].

Since the majority of SUMO in cells is present in the nucleus, much of the work undertaken to understand the role of sumoylation has focussed on its role in regulating events associated with DNA metabolism, such as the maintenance of chromatin structure, recombination and DNA damage responses [3,5,8,9]. More recently it has been demonstrated that sumoylation is required in the nucleolus to regulate ribosome biogenesis e.g. [35]. In order to obtain a fuller understanding of the role of sumoylation we have begun to investigate the protein-protein interactions and localisation of the mostly uncharacterised *S. pombe* SUMO protease, Ulp2. Our results from gel filtration and immunofluorescence studies indicate that Ulp2 is present in at least two high Mr complexes, which are distinct from the nuclear pore complex

that contains Ulp1. We demonstrate that it co-purifies with a number of proteins, many of which are involved in RNA metabolism or protein synthesis. We have investigated whether two of these proteins, eIF4G and eIF3h, are sumoylated, with the result that we observe SUMO modification of eIF4G but not eIF3h. Exposure of cells to conditions that lead to the formation of stress granules, results in increased sumoylation of eIF4G, and partial co-localisation of eIF4G and SUMO in the cytoplasm. Finally, we demonstrate that human eIF4G is sumoylated in HeLa cells, by both SUMO-1 and SUMO-2.

Materials and Methods

Strains and plasmids

The strains used in this work are described in Table 1. The strains containing myc-, HA or TAP-tagged *ulp1*, *ulp2*, *pli1*, *eIF4G* and *eIF3h* were created using the method of Bahler et al [36]. pREP41-His-SUMO was constructed by cloning the *pmt3* ORF into pREP41-His (created in this study). The *S. pombe* and human eIF4G and eIF4GI constructs, Sp C-term, N-FAG, M-FAG and C-FAG contain different fragments of the eIF4G/eIF4GI Orfs cloned into pET15b [37]. HeLa cell lines stably transfected with His-SUMO-1 and His-SUMO-2 were gifts from Prof R Hay (University of Dundee) [38,39].

Ulp2 expression and assay

The *ulp2* ORF was amplified from cDNA, by PCR and cloned into pFastBacHTa (GibcoBRL). Recombinant baculoviruses were generated according to GibcoBRL instructions. 50 ml infected cells were lysed in 50 mM Tris HCl pH 8, 5 mM β -mercaptoethanol, 1% nonidet, 1 mM PMSF. Ulp2 protein was purified using Talon resin. Ulp2 activity assays were conducted as described for Ulp1 [13].

Protein purification methods

His-tagged SUMO was recovered from *S. pombe* and human whole cell extracts under denaturing conditions with Ni^{2+} agarose beads. Cell extracts were prepared as follows: 10^8 cells (*S. pombe*) or $6\text{--}8 \times 10^6$ cells (Hela) were washed in ice cold water before being lysed by vortexing in 1.85 M NaOH, 7.5% v/v β -mercaptoethanol. The lysate was incubated on ice for 20 min after which TCA was added to a final concentration of 25%. Following a further 20 min incubation on ice, precipitated proteins were collected by centrifugation and resuspended and solubilised in 1 ml buffer A (6 M guanidinium HCl, 0.1 M NaH_2PO_4 , 10 mM Tris-HCl, pH 8). Insoluble material was removed by centrifugation. The cell extract was then incubated with Ni^{2+} agarose (Novagen) in Buffer A in the presence of 0.05% Tween-20, 150 mM imidazole. Purification on Ni^{2+} agarose was carried out according to the manufacturer's instructions. Samples were analysed by SDS-PAGE and Western blotting. His-tagged *S. pombe* and human eIF4GI fragments for *in vitro* sumoylation assays were purified from *E. coli* using Ni^{2+} agarose according to the manufacturer's instructions.

For gel filtration, 200 ml logarithmically growing cells were harvested, washed and then broken in 1 ml ice cold lysis buffer (45 mM HEPES pH 7.8, 300 mM KCl, 5 mM MgCl_2 , 5 mM EDTA, 5 mM EGTA, 12 mM NaF, 10% glycerol, 80 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, supplemented with Roche complete protease inhibitor). The extract was clarified by two rounds of centrifugation at 20,000 rpm for 10 min. 1.5 mg protein was loaded onto either a Superdex 200 or Superose 6 column pre-equilibrated in

Table 1. List of strains.

Strain	Genotype	Reference
Sp.011	<i>ade6-704, leu1-32, ura4-D18, h⁻</i>	[72]
Sp.611	<i>ulp1-myc:kan, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.614	<i>ulp2-myc:kan, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.658	<i>ulp1::ura4, ade6-704, leu1-32, ura4-D18, h⁻</i>	[13]
Sp.723	<i>pli1-myc:kan, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.874	<i>pmt3-GG:ura4, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
sp.851	<i>ulp1::ura4, pmt3-GG:ura4, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
sp.855	<i>ulp2::ura4, pmt3-GG:ura4, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.1470	<i>ulp2-TAP, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.2047	<i>eIF3h-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁺</i>	This study
Sp.2048	<i>ulp2-myc:kan, eIF3h-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.2068	<i>ulp2-myc:kan, eIF4G-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.2085	<i>ulp2::kan, ade6-704, leu1-32, ura4-D18, h⁻</i>	This study
Sp.2088	<i>eIF4G-HA:Nat, ade6-704, leu1-32, ura4-D18, h⁺</i>	This study

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lysis buffer. 0.5 ml fractions were collected and 15 µl of each was analysed by SDS PAGE.

For TAP-purification, 60 l *ulp2-TAP* cells were grown to mid-log phase, harvested and frozen at -80°C until required. Ulp2-TAP was purified using a modification of the method described by Seraphin et al. [40]. Specifically, the cells were broken in a 6850 freezer mill in 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 2 mM MgCl_2 , 0.1 mM NaF, 0.1% Nonidet NP40, 1 mM PMSF, 5 mM sodium orthovanadate, 80 mM β -glycerophosphate, 10 mM N-ethylmaleimide, supplemented with Roche complete protease inhibitor. All subsequent procedures were carried out at 4°C . The cell extract was centrifuged twice for 1 h at 10,000 rpm. Samples were pre-cleared by incubation with 200 µl Dynabeads for 30 min to remove proteins that bound non-specifically to the beads. The extracts were incubated with 300 µl IgG-coated Dynabeads for 2 h. The beads were collected and washed extensively before being resuspended in TEV buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT) with 250 units AcTEV protease (Invitrogen) for 3 h. The IgG-coated Dynabeads were removed from the preparation and Ulp2-TAP containing complexes were snap frozen in liquid nitrogen.

In vitro sumoylation assay

Recombinant His-tagged *S. pombe* eIF4G and human eIF4GI fragments were purified from *E. coli* and tested for sumoylation in an *in vitro* sumoylation assay as described elsewhere [41]. SUMO-TRGG (Pmt3-L109R,GG: the mature form of *S. pombe* SUMO containing a trypsin cleavage site immediately upstream of the diglycine motif) was used in the assay to facilitate the identification of the sumoylation sites by mass spectrometry.

Immunological methods

Western analysis was carried out as described previously [13]. Production of anti-SUMO and anti-eIF4GI (against the KRERK epitope) antisera has been described elsewhere [41,42], anti-myc antibodies for immunofluorescence were purified from cell supernatant (cell line CRL1729, from ATCC) using protein G-sepharose or were from Santa Cruz (sc-40), anti-HA antisera were from Santa Cruz (sc-7392) and monoclonal anti-tubulin antibodies

were from Sigma (T5168). Immunofluorescence was undertaken as described in Moreno et al. [43]. Cells were observed using an Applied Precision Deltavision Spectris microscope using deconvolution software.

Mass spectrometry

Complexes purified by purification of TAP-Ulp2 were analysed by SDS PAGE. Protein bands were visualised by staining with colloidal Coomassie, excised and subjected to trypsin in-gel digestion essentially as described by Schevchenko et al. [44]. The supernatant from the digested samples was removed and acidified to 0.1% TFA, dried down, and reconstituted in 0.1% TFA prior to LC MS/MS analysis. Each sample was loaded and desalted at a flow rate of 5 µl/min on a C18 trap column (200 µm ID x 1 cm, 5 µm PepMap 100, Dionex) in buffer A (acetonitrile (2% v/v): water (97.9% v/v): formic acid (0.1% v/v)). The tryptic peptides were fractionated on a C18 reverse phase column (75 µm ID x 25 cm, 3 µm PepMap 100, Dionex) using an Ultimate U3000 nano-LC system (Dionex) and a 2 hr linear gradient from 95% buffer A to 50% buffer B (acetonitrile (95% v/v): water (4.9% v/v): formic acid (0.1% v/v)) at a flow rate of 300 nl/min. Eluted peptides were directly analysed by tandem mass spectrometry using a LTQ Orbitrap XL hybrid FTMS (ThermoScientific) operated in parallel acquisition IDA mode with nominal resolution of 60,000 (FWHM) at m/z 400 for MS1 and the top six most abundant multiply charged ions being selected for CID fragmentation in the linear ion trap followed by dynamic exclusion for 90 secs.

Derived MS/MS data were searched against the *S. pombe* subset of the UniProt Knowledgebase release 15.13 database using Sequest version SRF v. 5 as implemented in Bioworks v 3.3.1 (Thermo Fisher Scientific), assuming carboxyamidomethylation (Cys), deamidation (Asn and Gln) and oxidation (Met) as variable modifications and using a peptide tolerance of 10 ppm and a fragment ion tolerance of 0.8 Da. One missed cleavage was allowed and filtering criteria used for positive protein identifications were Xcorr values greater than 1.9 for +1 spectra, 2.2 for +2 spectra and 3.75 for +3 spectra and a delta correlation (DCn) cut-off of 0.1.

For the identification of sumoylation sites, reduction and alkylation were instead performed using TCEP and MMTS respectively as previously described [45] and bioinformatics analysis following conversion of LTQ-Orbitrap (.raw) raw data files to Mascot generic format (MGF) via Mascot Distiller (Matrix Science) performed essentially as described by Chicooree et al. [46] using the MASCOT search engine with the UniProt Knowledgebase release 15.13 database with the *S. pombe* subset as selected taxonomy. Precursor ion tolerances were again set at 10 ppm and MS/MS peptide ion tolerance to 0.8 Da, and the same variable modifications assumed. However, two missed trypsin cleavages were allowed.

Following trypsin digestion, cleavage of the SUMO moiety was expected to leave a Gly-Gly isotag on modified residues. The GG isotag (on lys) was accordingly also searched as a variable modification. Following MASCOT searches, putative sites of SUMOylation were noted and the relevant raw MS/MS spectra subsequently examined manually to confirm presence of the modification (the GG isotag).

Results

Biochemical characterisation of *S. pombe* Ulp2

A comparison of the *S. pombe* Ulp2 sequence was made with those of the two *S. cerevisiae* SUMO proteases, Ulp1 and Ulp2 [47]. Since *S. pombe* Ulp2 more closely resembles *S. cerevisiae* Ulp2 (required solely for deconjugating SUMO from high Mr SUMO-containing species) than it does Ulp1 (which is required for both processing and deconjugating), it is likely that the main activity of *S. pombe* Ulp2 is in deconjugating SUMO from sumoylated targets rather than in processing SUMO to the mature form. Before proceeding to analyse the localisation or protein-protein interactions of Ulp2, we first confirmed its proposed biochemical activity. His-tagged Ulp2 was purified from insect cells as described in Materials and Methods. Using assays we described previously [13], we demonstrate that Ulp2 is significantly less able than Ulp1 to process SUMO to the mature form (Figure 1A, lane 2 (Ulp1) and lane 3 (Ulp2)), but is capable of deconjugating SUMO from high Mr species in an N-ethylmaleimide- (NEM)-dependent manner (Figure 1B). These results confirm that like *S. cerevisiae* Ulp2, *S. pombe* Ulp2 is a cysteine protease whose main function is in deconjugating SUMO from target proteins.

Deletion of the *ulp2* gene results in a severe growth defect and sensitivity to a range of stresses

Deletion of *pmt3* (which encodes SUMO), *hus5* (the gene encoding the SUMO-conjugating enzyme, E2), *rad31* (which encodes one sub-unit of the SUMO activating enzyme, E1) or *ulp1* (another SUMO-specific protease gene) results in severe growth and morphological abnormalities [13,48–50]. We therefore wished to determine whether disrupting the *ulp2* gene has any effect on cell growth or viability. Disruption of the gene is not lethal. However, *ulp2-d* cells form very small colonies and show distinct morphological abnormalities resembling *hus5* and *rad31* mutants (data not shown). Comparison of SUMO-containing species in *ulp1-d* and *ulp2-d* cells (Figure 1C, lanes 3 and 4) supports the notion that the main function of Ulp2 is in the removal or dismantling of high Mr SUMO-containing species, rather than in processing precursor SUMO. Provision of the mature form of SUMO (Pmt3-GG) in *ulp1-d* cells (lane 5) results in the incorporation of SUMO into high Mr species (unlike the situation in *ulp1-d* cells, lane 3), while in *ulp2-d* cells (lane 6), the level of high Mr species is slightly increased.

To begin to identify cellular processes involving Ulp2, we tested whether *ulp2-d* cells are sensitive to the DNA synthesis inhibitor, hydroxyurea (HU) and other stresses (Figure 1D), and compared these responses to those of *ulp1-d.pmt3-GG* cells (where the mature form of SUMO is provided, so that cells are only defective in the deconjugating activity of Ulp1). Since *ulp2-d* and *ulp1-d.pmt3-GG* cultures contain a high proportion of dead cells, it was necessary to plate more cells for these strains compared to wild type (approximately 10 fold). These data indicate that *ulp2-d* cells are temperature sensitive, unlike the *ulp1-d.pmt3-GG* strain, but similar to the *S. cerevisiae* *ulp2Δ* strain [51], and sensitive to the DNA synthesis inhibitor, hydroxyurea (HU, 2 mM). They are also sensitive to the protein synthesis inhibitor, cycloheximide (CHX, 10 and 20 µg/ml) and KCl (1 M) indicating that Ulp2 likely has roles in numerous cellular processes.

Ulp2 is present in high molecular weight complexes

Throughout most of the cell cycle, Ulp1 is associated with the nuclear envelope [13], and specifically with the nuclear pore complex [52,53]. To determine whether Ulp2 is also part of a high Mr complex we undertook gel filtration analysis. Figure 2A indicates that, as expected, Ulp1 elutes in the void volume, consistent with it being present in a high Mr complex. Ulp2 also elutes in the void volume like Ulp1, but additionally, it is present in fractions corresponding to an approximate Mr of 670 kDa. This suggests that Ulp2 is likely to be present in at least two different complexes. In contrast to the results obtained for Ulp1 and Ulp2, Pli1, an E3 SUMO ligase [54], does not elute in these high Mr fractions, implying that it likely exists in cells as a monomer or possibly a dimer.

Ulp2 is located in both the nucleus and cytoplasm, but is predominantly nuclear

Since a proportion of Ulp2 co-elutes with Ulp1 in the void volume, we wished to determine whether some or all of the Ulp2 co-localises with Ulp1 in cells, i.e. is at the nuclear periphery. We therefore analysed the localisation of Ulp2. Figure 3 indicates that Ulp2 is present in foci that are predominantly nuclear, with a small proportion in the cytoplasm. Little if any Ulp2 is located at the nuclear periphery. Thus the location of Ulp2 is distinct from that of Ulp1 [13], indicating that it is unlikely to be part of nuclear pore complexes. In many cases, Ulp2 co-localises with SUMO. Ulp1 undergoes distinct changes in localisation during the cell cycle, its localisation changing from the nuclear periphery where it is for most of the cell cycle, to the region between the separating DNA masses during mitosis [13]. In contrast, the location of Ulp2 appears to be relatively unchanged in cells at different cell cycle stages. For example, during mitosis (Figure 3, TRITC panel, cells labelled 4), a time when Ulp1 relocates, the distribution of intranuclear Ulp2 foci is very similar to that observed at other times in the cell cycle (cells labelled 1–3) and is unchanged.

Ulp2 co-purifies with proteins associated with RNA metabolism and protein synthesis

To begin to identify the nature of the complexes observed in Figure 2, we C-terminally-tagged Ulp2 with TAP in the genome (*ulp2-TAP*) and isolated the tagged protein and associated proteins as described in Materials and Methods. Protein complexes were analysed by SDS PAGE (Figure 4) and fractions excised from the gel for mass spectrometric analysis. As shown in Table S1 and Table 2, the majority of the proteins identified are associated with RNA metabolism, such as RNA processing, ribosome biogenesis or initiation of translation. To ensure that these proteins co-

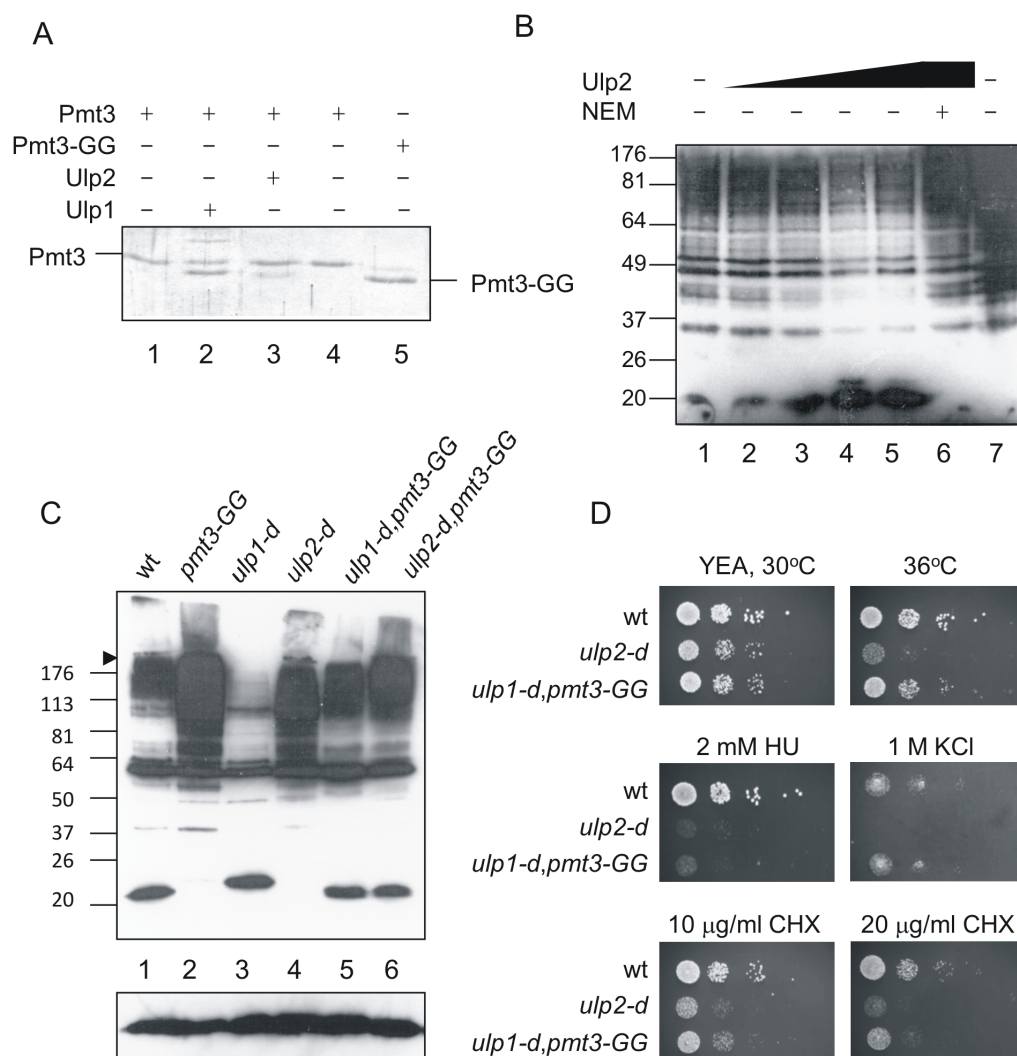


Figure 1. Analysis of Ulp2 function. A. Assay for SUMO-processing activity. Lanes 1–4 contain full length SUMO, lane 5 SUMO-GG. Lanes 1,5, unincubated controls, lanes 2–4 were incubated at 20°C for 2 h following addition of 0.72 µg Ulp1 (lane 2), 2.32 µg Ulp2 (lane 3) or 2 µl buffer (lane 4). Proteins were analysed by SDS PAGE followed by staining with Coomassie Brilliant Blue. B. Assay for de-conjugating activity. *S. pombe* cell extracts were prepared using standard native extraction procedures. Extracts were incubated at 20°C for 2 h (lanes 1–6), lane 1 5 µl of fraction from extract from *E. coli* cells transformed with empty vector, equivalent in volume to the Ulp2-containing fraction from *ulp2*-transformed cells, lane 2 0.6 µg Ulp2, lane 3 1.2 µg Ulp2, lane 4 2.4 µg (5 µl) Ulp2, lane 5 4.8 µg Ulp2, lane 6 1.2 µg Ulp2 pre-incubated with 5 mM NEM, lane 7 total cell extract without incubation at 20°C. Assays were analysed by Western blotting with anti-SUMO antisera. C. Western analysis of total cell extracts using anti-SUMO antisera. Both the separating and stacking gels (6% polyacrylamide in the stacking gel) were blotted. D. Ten microlitre of 10 fold serial dilutions of cells were plated onto YEP agar plates with or without additives as indicated. 10x amount of cells of *ulp2-d* and *ulp1-d,pmt3-GG* were used compared to wild type.

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purified specifically with Ulp2, a parallel purification was undertaken using Rad9-TAP, and from cells expressing the TAP tag alone (Figure S1). Rad9 is a member of the 9-1-1 complex required for the DNA integrity checkpoint [55], and would not be expected to interact with a the same proteins as those that interact with Ulp2. Very little protein co-purified with the TAP-tag alone, while purification of Rad9-TAP yielded a quite different set of bands. Most of the proteins co-purifying with Rad9 were associated with DNA metabolism as expected (data not shown) and only one protein, glyceraldehyde 3-phosphate dehydrogenase, was common to the Ulp2-TAP and Rad9-TAP preparations.

A number of proteins required for ribosome biogenesis, including some of those we identified by mass spectrometry, have recently been demonstrated to be sumoylated (Table 2) [6,7,23–

27,56–58]. However, little is known about the effect of sumoylation on the function of translation factors. We therefore selected two translation initiation factors, eIF4G and eIF3h for further study. The analysis of some of the other factors will be described elsewhere. eIF4G has been well characterised in *S. cerevisiae* and mammalian cells [18,22] and to some extent in *S. pombe* [59]. eIF4G acts as a scaffold protein as part of the eIF4F complex to recruit mRNA to the ribosome for translation [21], while eIF3h is a non-core subunit of the eIF3 complex linking eIF4F/mRNA to the ribosome in mammalian cells [60]. Gel filtration analysis of whole cell extracts from cells containing Ulp2-myc and either eIF4G-HA or eIF3h-HA indicates that the majority of eIF4G co-elutes with Ulp2 (Figure 2B). In contrast, eIF3h elutes in multiple

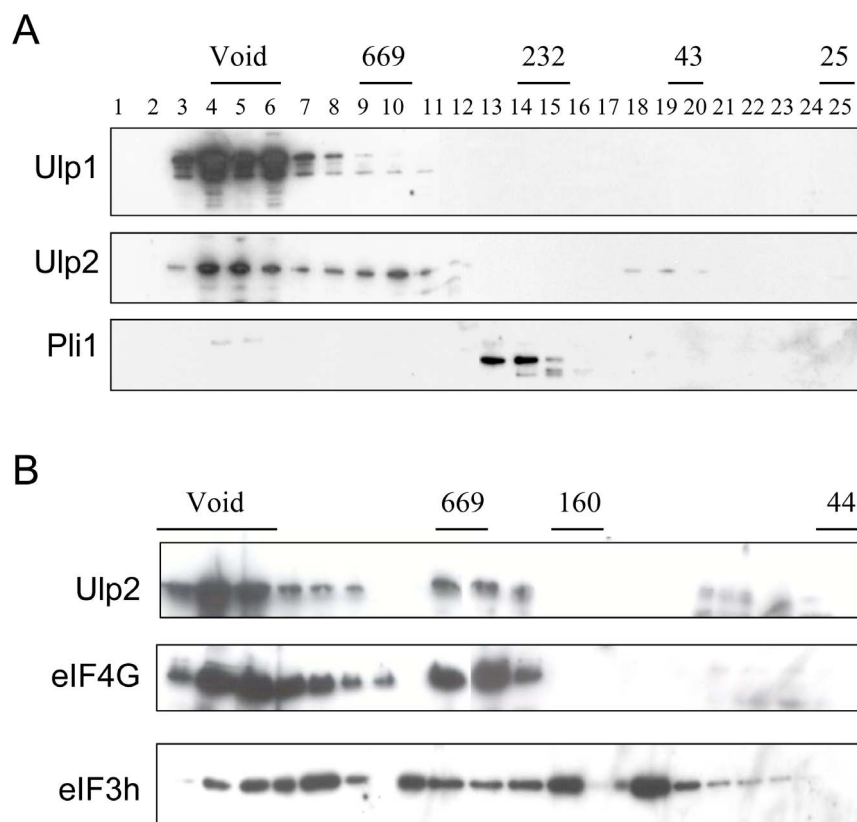


Figure 2. Ulp2 is present in high Mr complexes. Analysis of complexes by gel filtration. A. Total cell extracts from *ulp1-myc*, *ulp2-myc* or *pli1-myc* strains were analysed on a Sephadex 200 column, and fractions were western blotted with anti-myc antibodies. B. Total cell extracts from *ulp2-myc,eIF4G-HA* and *ulp2-myc,eIF3h-HA* strains were analysed on a Superose 6 column, fractions were western blotted with anti-myc and anti-HA antibodies.

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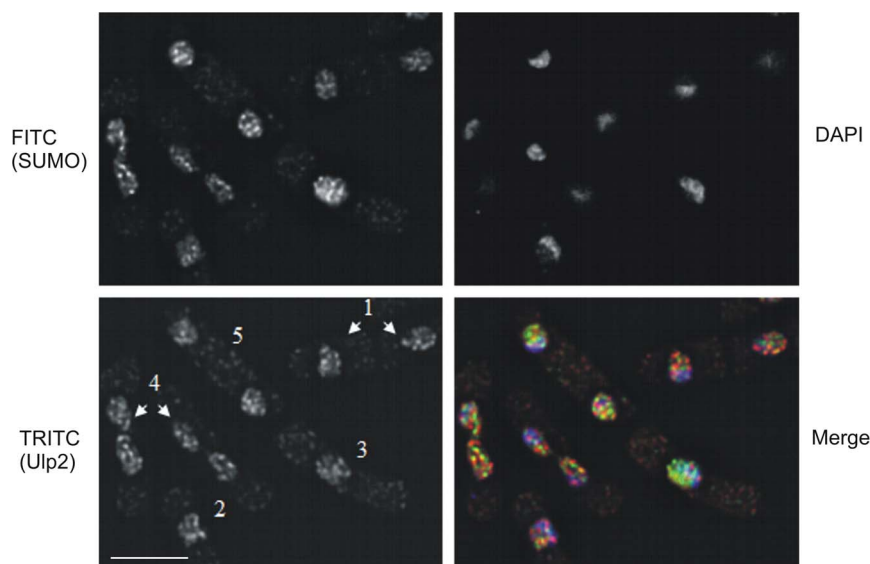


Figure 3. Ulp2 is localised predominantly within the nucleus. A. Cells containing myc-tagged *ulp2* as the sole copy of the *ulp2* gene were incubated with anti-myc antisera (mouse monoclonal) and anti-SUMO antisera (rabbit polyclonal) followed by TRITC-conjugated anti-mouse IgG antisera, FITC-conjugated anti-rabbit IgG antisera and DAPI. Merge = overlay of TRITC (red), FITC (green) and DAPI (blue) staining. 1: early G2 cells, 2,3: late G2 cells, 4: mitotic cells, 5: S phase cells. Bar = 5 μm.

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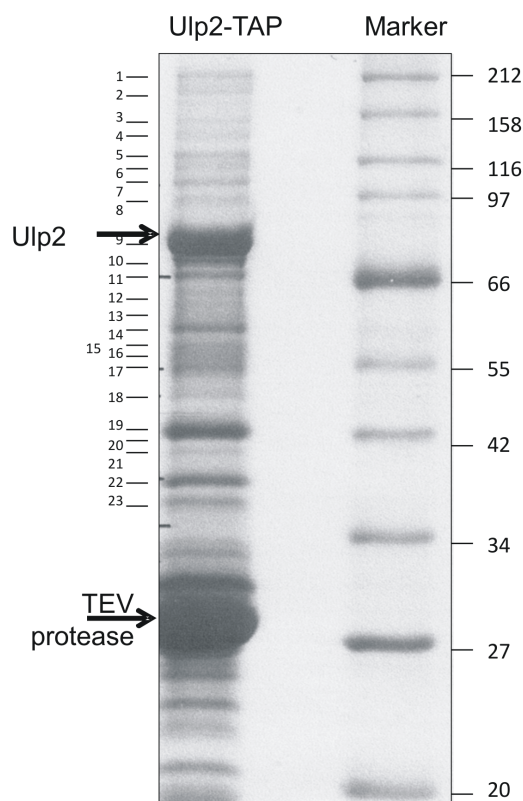


Figure 4. Purification of Ulp2-TAP. SDS-PAGE of Ulp2-Tap and associated proteins. TEV = TEV protease, used to cleave Ulp2 from TAP tag. Numbers refer to gel slices analysed by mass spectrometry. doi:10.1371/journal.pone.0094182.g004

fractions, suggesting it is present in several different sized complexes.

eIF4G, but not eIF3h, is sumoylated in *S. pombe*

One possibility to explain the interaction of eIF4G and eIF3h with the SUMO protease Ulp2 is that they are themselves modified by SUMO. In order to determine whether this is the case, cells containing genomic copies of HA-tagged eIF4G or eIF3h were co-transformed with pREP41-His-SUMO. His-tagged SUMO was purified on Ni²⁺ agarose. Denaturing conditions (with 6 M guanidinium HCl in the binding buffer, followed by 6 M urea, 300 mM imidazole washes) were used to ensure that sumoylation of the individual proteins was being observed, rather

than that of other components of the eIF4F or eIF3 complexes). Figure 5A, shows that eIF4G is specifically recovered in the presence of His-tagged SUMO (lane 1), but not in the absence of His-tagged SUMO (lane 2), indicating that it is sumoylated in *S. pombe*. In contrast, eIF3h is not recovered in either the absence or presence of His-tagged SUMO (Figure 5B), indicating that this translation factor is not sumoylated in fission yeast. Its co-purification with Ulp2 may thus be through the interaction of Ulp2 with other member(s) of the eIF3 complex.

Conditions that induce stress granules affect the localisation and sumoylation of eIF4G

Since one of the functions of sumoylation is to affect protein localisation, we next investigated whether eIF4G and SUMO co-localise. Figure 6 shows that in untreated cells, as has been shown previously [61], the majority of eIF4G is cytoplasmic as expected for a translation initiation factor. As has been observed in *S. cerevisiae* and human cells [62,63], a small amount of eIF4G is also present in the nucleus, where it is proposed to couple RNA processing events in the nucleus with translation in the cytoplasm. In contrast to the situation with eIF4G, the majority of the SUMO protein is present in the nucleus (Figures 3 and 6). We observe that a significant proportion of the nuclear eIF4G co-localises with SUMO, suggesting sumoylation of eIF4G may have a role in regulating RNA processing or localisation.

Protein synthesis can be inhibited by a variety of factors. For example, cycloheximide (CHX) interacts with ribosomes and inhibits the elongation step, while exposure of *S. pombe* cells to 1 M KCl inhibits protein synthesis by the sequestration of translation initiation factors and mRNA into cytoplasmic stress granules [64]. Following treatment with CHX, eIF4G staining is slightly more punctate than in untreated cells, while the pattern of SUMO staining is unchanged. In these cells, there is a low level of colocalisation of eIF4G and SUMO in the nucleus. Interestingly, exposure of cells to CHX results in distorted nuclei. The reason for this is not known, but it could be due to disruption of RNA processing and/or localisation by CHX.

In *S. pombe* and mammalian cells eIF4G and eIF4GI respectively, are among the translation factors present in stress granules [61,65,66]. To investigate stress granule formation in *S. pombe*, we exposed cells to 1 M KCl. In these cells, eIF4G is present in fewer, but quite bright, punctate cytoplasmic foci (Figure 6). This pattern of staining is similar to what has been observed for stress granules in *S. pombe*, and in particular, what has previously been observed for eIF4G in this organism [61,64]. In these cells, there was occasional co-localisation of the two proteins in the cytoplasm and this appeared to reflect the appearance of eIF4G and SUMO in the same granule.

Table 2. Summary of proteins identified by mass spectrometry that co-purified with TAP-Ulp2.

Function	Protein
Translation	eIF2 α , eIF2 β , eIF3a, eIF3b, eIF3c, eIF3 γ , eIF3h, eIF4G, EF1 α , EF2B, eEF3B, EF2, Pabp
RNA synthesis	Rpa1, Rpa2,
RNA processing	Rrp5, SPAC694.02, Exo2, Dhp1, Upf1, SPBC19G7.10C, Nop2, Dbp2, Prp19, Sla1,
Ribosome biogenesis	aconitate hydrolase/mitochondrial ribosomal protein subunit L49, SPAC22G7.05, SPAC1142.04(Noc2 predicted), Hsc1/Sks2, Rpl301, Rpl302, Rml2
DNA metabolism	Tcg1, Rfc5,
Other	Pfk1, SPBC16h5.12C, glutamate 5-kinase (predicted), Gpd1, Gpd3

Data from [6,7,23–26,56–58].

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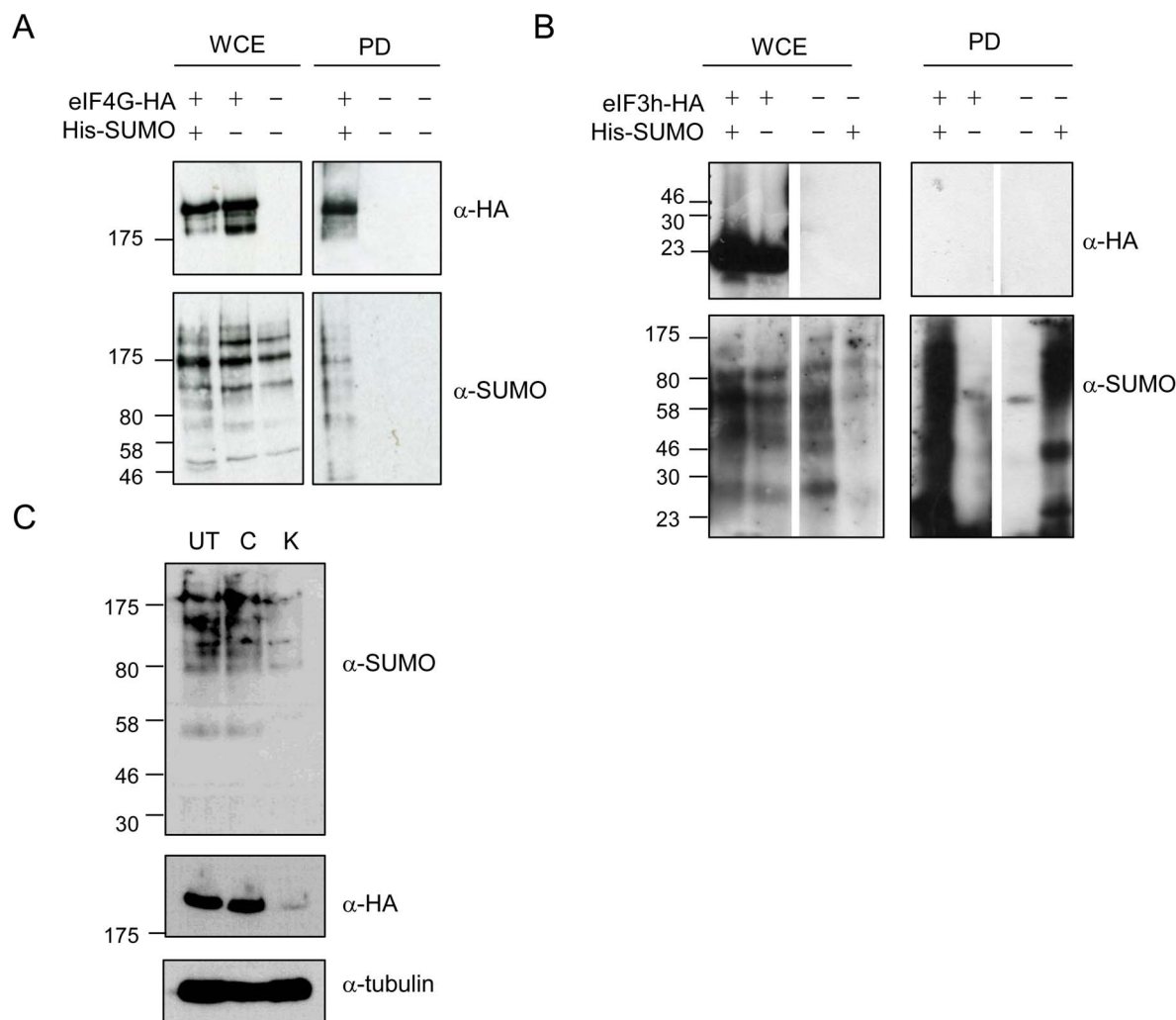


Figure 5. eIF4G, but not eIF3h, is sumoylated. His-tagged SUMO was expressed in cells containing genomically tagged (HA) copies of eIF4G (A) and eIF3h (B). WCE = whole cell extract, PD = Ni^{2+} -agarose pull down. Blots were probed with anti-HA or anti-SUMO antisera. C. Western blot of whole cell extracts from cells containing genomically tagged eIF4G-HA. UT = untreated, C, K = incubated for 30 min with 100 $\mu\text{g}/\text{ml}$ CHX (C) or 1 M KCl (K). doi:10.1371/journal.pone.0094182.g005

Another protein known to be present in stress granules is polyA-binding protein (PABP) [61]. We therefore compared the localisation of eIF4G and PABP in cells exposed to 1 M KCl. We observe PABP in large cytoplasmic granules, which are different to those we observe in cells only containing HA-tagged eIF4G-HA (Figure S2A and Figure 6). Curiously, in some of the cells that contain both eIF4G-HA and PABP-RFP, eIF4G is now also present in large granules where it co-localises with PABP. Further analysis of PABP-RFP containing cells indicated that a proportion of the SUMO is mislocalised to the cytoplasm (Figure S2B). This suggests that C-terminal RFP-tagging of PABP may affect its function and/or localisation.

Following exposure to 1 M KCl, we noticed that there was less staining of both eIF4G and SUMO compared to that in untreated cells. Western analysis of eIF4G and SUMO levels indicates that in response to 1 M KCl the levels of both proteins are significantly reduced (Figure 5C). The reason for this is unknown, but may be due to the fact that a proportion of the eIF4G and SUMO is insoluble and not recovered in the extract. Alternatively, and in

our view the more likely explanation, we propose that in response to this stress, there is increased proteolysis of both proteins.

We next investigated whether sumoylation of eIF4G is affected by exposure of cells to either CHX (100 $\mu\text{g}/\text{ml}$) or KCl (1 M). Figure 7A indicates that there is an increase in sumoylation in response to KCl, with levels of sumoylation unaffected by exposure to CHX, when compared to levels in untreated cells (with relative levels being 1:1:1.5; wt, CHX-treated, KCl-treated, respectively). These data suggest that sumoylation of eIF4G may be associated with stress granule formation and/or proteolysis of the translation initiation factor.

Human eIF4GI is sumoylated

In order to analyse the role of sumoylation of *S. pombe* eIF4G we investigated the possibility of testing the protein for ability to be sumoylated in our *in vitro* sumoylation assay, as this could help us identify the sumoylated lysine residue(s). However, two factors make this identification difficult. Firstly, in order to purify protein for an *in vitro* sumoylation assay, we would need to clone the full length *S. pombe* eIF4G cDNA. We have previously observed that

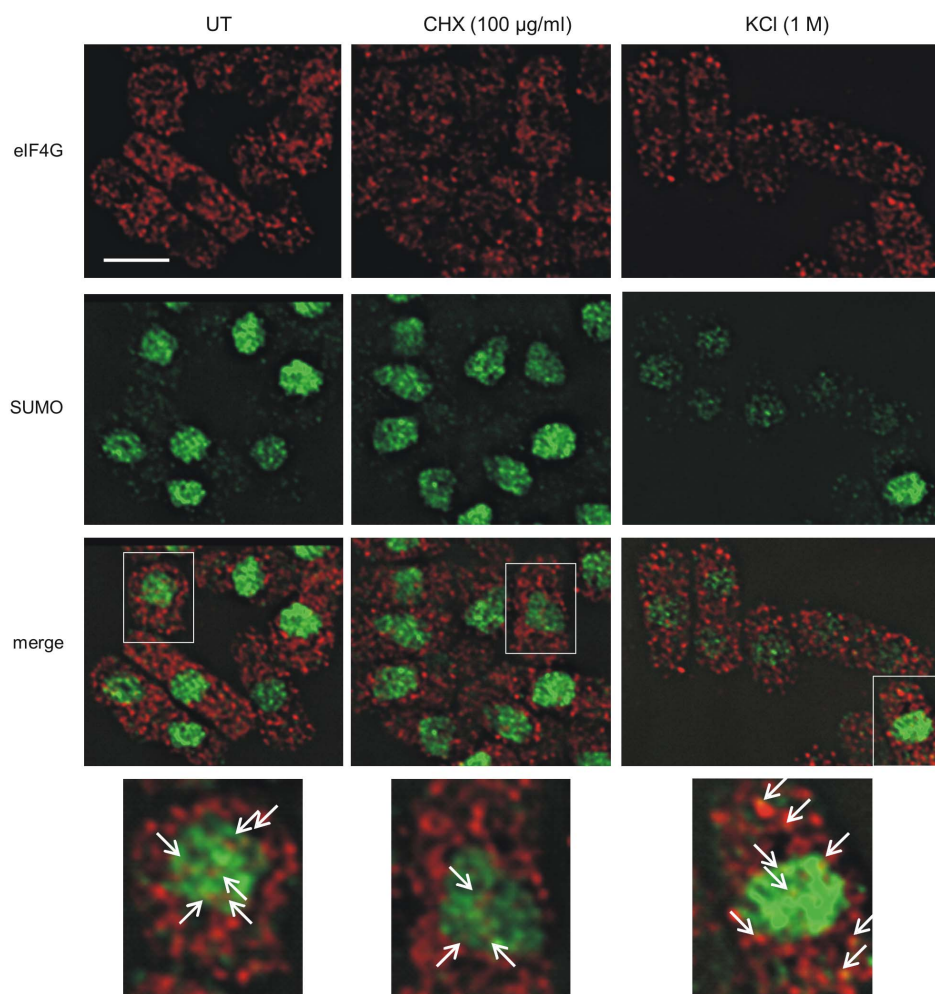


Figure 6. Effect of cycloheximide and KCl on localisation of eIF4G and SUMO. Cells containing eIF4G-HA, untreated (UT) or exposed to CHX (100 µg/ml) or KCl (1 M) as indicated, were incubated with anti-SUMO antisera (green) and anti-HA antisera (red). Bar = 5 µm. Bottom panel, regions indicated by boxes in panel above. Arrows indicate sites of colocalisation of SUMO and eIF4G.
doi:10.1371/journal.pone.0094182.g006

plasmids containing the N-terminus of the *S. pombe* eIF4G coding sequence cannot be tolerated in *E. coli* [59], so that full length eIF4G cannot be expressed in *E. coli*. The reason for this is unknown, but may be due to the presence of a highly repeated sequence within the eIF4G coding sequence. Secondly, this highly repeated sequence (present in the coding sequence in the *S. pombe*, but not in the *S. cerevisiae* or human proteins) contains 16 repeats of a perfect sumoylation site consensus motif (AKRE), which would likely make identification of the site(s) difficult, even if we were able to express the full length protein. We therefore expressed a C-terminal fragment (comprising aa 970–1403), which contains eIF4E, eIF4A and eIF3 binding sites (Figure 7B) and tested this in our *in vitro* sumoylation assay. We did not observe any sumoylation of this fragment, implying that sumoylation likely occurs in the N-terminus of the protein.

To further analyse the role of sumoylation we set out to determine whether human eIF4GI is sumoylated and if so, to identify the sumoylation site(s) in this protein. We used HeLa cell lines stably transfected with either His-SUMO-1 or His-SUMO-2 [38,39]. His-tagged SUMO was recovered from cell extracts prepared under denaturing conditions. Figure 7C indicates that eIF4GI is not recovered from extracts of cells that do not contain His-tagged SUMO (lane1), but is isolated from extracts of cells

containing His-SUMO-1 (lane 2) and to a lesser extent from cells expressing His-SUMO-2 (lane 3). This confirms that, like *S. pombe* eIF4G, human eIF4GI is sumoylated.

We next sought to identify the sumoylation sites on human eIF4GI. In order to facilitate our analysis, we used three different human eIF4G fragments, N-FAG, M-FAG and C-FAG (Figure 7B, [37]). These protein fragments were purified from *E. coli* and tested in our *in vitro* sumoylation assay (data not shown). Slow migrating forms of eIF4G were excised from gels and analysed by mass spectrometry. Two sumoylation sites were identified: K1368 and K1588 (Figure 7D). These map to two domains of eIF4GI which interact with eIF4A and the protein kinase, Mnk1, respectively [18,21,22]. These results suggest that sumoylation may affect the interaction of eIF4GI with these two proteins.

Discussion

In order to analyse the role of *S. pombe* Ulp2, we purified Ulp2-TAP-containing complexes. We identified proteins involved in RNA synthesis or processing, ribosome biogenesis and translation. This is consistent with recent reports that a number of proteins required for ribosome biogenesis and RNA processing are sumoylated [6,67,68]. While this manuscript was in preparation,

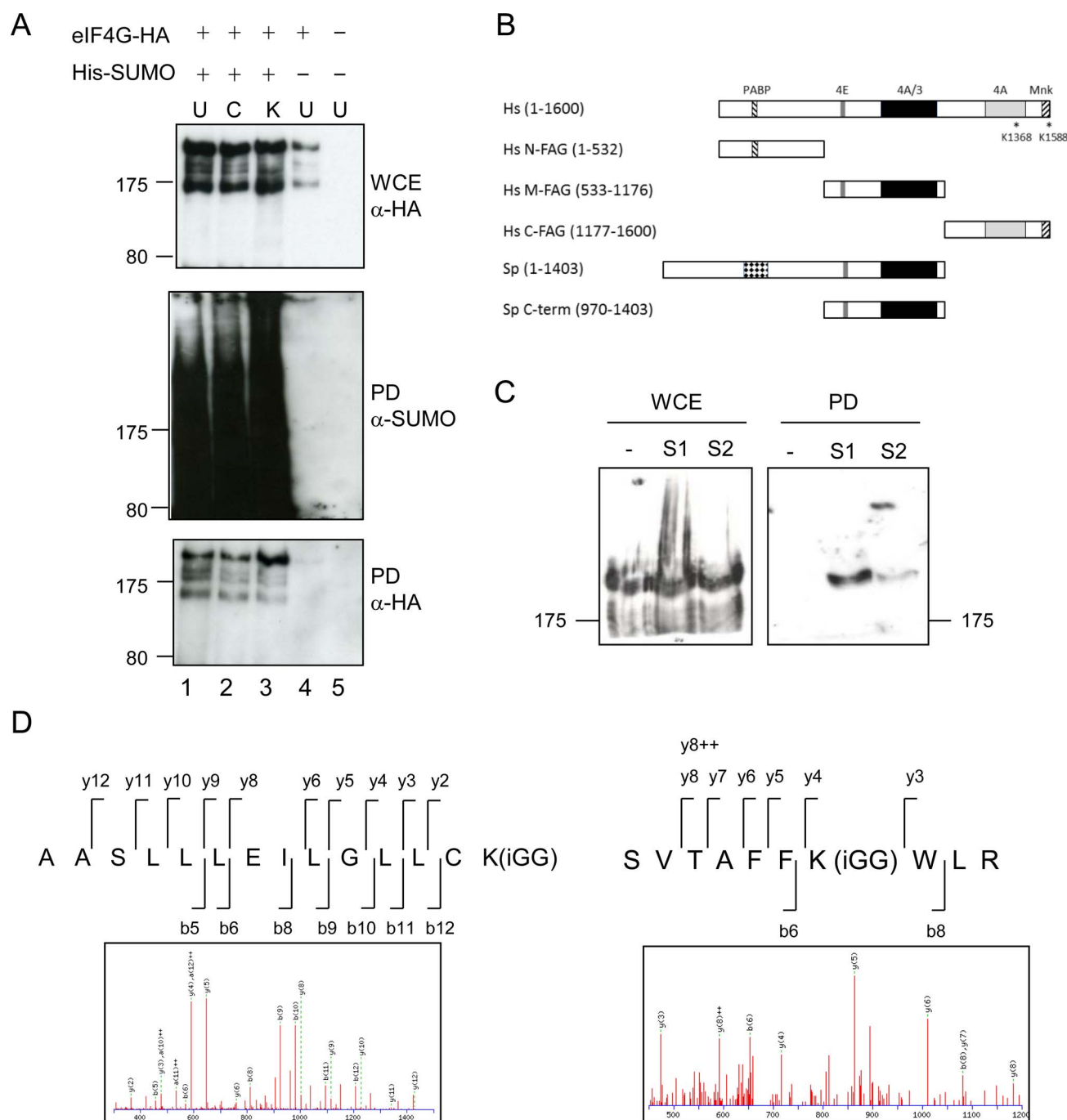


Figure 7. Human eIF4G is sumoylated. A. *S. pombe* cells containing His-tagged SUMO and HA-tagged eIF4G as indicated were treated with CHX (100 μ g/ml) or KCl (1 M), and His-tagged SUMO pulled down, and analysed as in Figure 5. B. Comparison of human and eIF4G proteins, indicating protein binding domains: PABP = polyA binding protein, 4E = eIF4E, 4A = eIF4A, 3 = eIF3, Mnk = MAP kinase-interacting kinase 1. C. Whole cell extracts (WCE) and Ni^{2+} pull-down (PD) from extracts of HeLa cells stably transfected with His-tagged SUMO-1 (S1) or SUMO-2 (S2) or nothing (-). Western blots probed with anti-eIF4G1 (KRERK epitope) antisera. D. Representative eIF4G ion mass spectra (MS/MS spectra) showing identification of the *in vitro* sites of sumoylation.

a global analysis of the SUMO system interactome in *S. cerevisiae* identified a range of proteins including a number required for ribosome biogenesis and rRNA processing that interact with Ulp2 [69]. Additionally, the nucleolar SUMO-specific protease, SENP3, has been demonstrated to reverse the SUMO modification of nucleophosmin to be required for rRNA processing [70].

Although a number of translation factors, required for both the initiation and elongation steps of protein synthesis, have been identified in global screens as being sumoylated e.g. [23–28,30,31], little is known about the role of sumoylation of these proteins. This is in contrast to the situation with the role of sumoylation in ribosome biogenesis. We therefore focussed our attention on two *S. pombe* translation initiation factors in our list of Ulp2-interactors:

eIF4G and eIF3h. Both proteins are known to be present in high Mr complexes, with eIF4G being part of the eIF4F complex while eIF3h is part of the eIF3 complex [22]. We demonstrate here that eIF4G, but not eIF3h is sumoylated *in vivo*. These results are supported by the genome-wide analyses of sumoylated proteins that have been undertaken, that indicate that eIF4G is sumoylated [24,28] but which have not to date identified eIF3h as a sumoylation target.

As the most prominent role of translation initiation factors is in cytoplasmic protein synthesis, we began by investigating whether Ulp2 is associated with polysomes. However, we observed that while Ulp2 migrated at the same position in sucrose gradients as polysomes, it was still present in these fractions under conditions (2.5 mM EDTA) where polysomes were disrupted, indicating that the majority of Ulp2 is not associated with actively translating polysomes (data not shown). This result confirms our gel filtration analysis and localisation studies, and indicates that Ulp2 is present in very high molecular weight complexes, but discounts the possibility that Ulp2 is associated with actively translating polysomes.

The role of sumoylation of translation factors has not been well studied, apart from that of eIF4E [29,33]. eIF4E is an mRNA cap-binding protein, and one of the proteins that interacts with eIF4G to form the eIF4F complex [22]. eIF4E is regulated by phosphorylation and by interaction with eIF4E-binding proteins (4E-BPs). Sumoylation of eIF4E on five lysines is promoted by its phosphorylation at S209, and results in its dissociation from 4E-BP1. Sumoylation did not interfere with mRNA recognition but enhanced eIF4F complex assembly on the mRNA cap, promoting the expression of ornithine decarboxylase, c-myc and Bcl-2, driving the anti-apoptotic and oncogenic activity of eIF4E [33]. As phosphorylation of eIF4E has been shown to play a role in selective nuclear export of mRNA [71], it is likely that sumoylation of eIF4E occurs in the nucleus and/or as it emerges into the cytoplasm.

We have shown that in response to osmotic stress (1 M KCl), conditions that induce stress granules in fission yeast, the overall levels of SUMO and eIF4G are reduced. We have also shown that under these conditions, there is increased sumoylation of eIF4G. The role of this modification is not known. Our results suggest two possible scenarios: the first being that sumoylation is targeting eIF4G for degradation, possibly via the action of a SUMO-targeted ubiquitin ligase (STUbL). The second possibility is that sumoylation may be targeting eIF4G to stress granules. Further work is needed to distinguish between these two possibilities.

The two sumoylation sites in human eIF4GI that we have identified are not conserved in fission yeast eIF4G, as this protein lacks the C-terminal domains present in human eIF4GI (Figure 7B). Their positions suggest that sumoylation of this protein may be affecting interactions of eIF4GI with eIF4A and Mnk1. eIF4A is a DEAD-box protein that participates in translation initiation and binds to eIF4GI [18,21,22]. Functioning as an ATP-dependent RNA helicase, eIF4A is believed to unwind secondary structure in the 5'-untranslated region of mRNAs to enable ribosome scanning. The RNA-stimulated ATPase and ATP-dependent helicase activities of eIF4A are enhanced by its

interaction with two domains on eIF4GI, one in the C-terminus and one in the middle domain [18,22]. Interaction and subsequent recycling of eIF4A from the eIF4G/eIF4A complex stimulates the eIF4A helicase activity required for the mRNA scanning process. It is possible that sumoylation of eIF4GI either directly or indirectly affects the interaction with eIF4A, thereby regulating translation initiation. Mnk1 is a kinase which binds at the extreme C-terminus of eIF4GI and regulates the phosphorylation of eIF4E at Ser209 [18,21,22]. Phosphorylated eIF4E has been shown to be modified by sumoylation on five lysine residues [33] promoting eIF4F complex formation and specific protein synthesis [33]. Sumoylation of K1588 on eIF4GI could prevent the binding of Mnk1, reduce eIF4E phosphorylation and thereby abrogate sumoylation of eIF4E and specific mRNA translation. As phosphorylation of eIF4E is associated with tumour cell formation and increased resistance of tumour cells to apoptosis, sumoylation of eIF4GI at this site could provide a novel and undiscovered mechanism to regulate cell growth and proliferation in mammalian cells. Further work needs to be done to address this.

In conclusion, our results demonstrate that *S. pombe* and human eIF4GI are both sumoylated, and that in *S. pombe* this modification is increased under conditions that promote the formation of stress granules. We have also identified the target lysine residues that are used for sumoylation *in vitro* in human eIF4GI. It will be of interest to determine whether these sites are also used *in vivo*, and to identify the role of this sumoylation.

Supporting Information

Figure S1 Comparison of proteins co-purifying with Ulp2-Tap and Rad9-Tap. Extracts from cells expressing Ulp2-Tap, Rad9-Tap (Methods S1) or Tap alone were subjected to the same purification procedure and analysed by SDS-PAGE followed by staining with colloidal coomassie. (TIF)

Figure S2 Colocalisation of eIF4G with PABP. A. Strain containing eIF4G-HA and PABP-RFP stained with anti-HA and anti-RFP antisera. Secondary antisera: anti-rabbit FITC conjugated, anti-mouse TRITC-conjugated. B. Strains containing either eIF4G-HA or Pabp-RFP (Methods S1) as indicated, stained with anti-SUMO antisera. (TIF)

Table S1 Identity of proteins co-purifying with Ulp2-TAP. Proteins identified by LC MS/MS (Methods S1). (DOCX)

Methods S1
(DOCX)

Author Contributions

Conceived and designed the experiments: FZW SJM DLT. Performed the experiments: JJ MF LZ OW LS RB DS LDB FZW DLT. Analyzed the data: MF DS LDB SJM FZW. Contributed reagents/materials/analysis tools: JJ MF LZ LS RB DS LDB SJM FZW. Wrote the paper: FZW SJM.

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Weighing up the possibilities: Controlling translation by ubiquitylation and sumoylation

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Keywords:

Abbreviations: eIF, eukaryotic initiation factor; PABP, poly(A) binding protein; 4E-BP, eIF4E-binding protein; mTORC, mechanistic target of rapamycin; Ubl, ubiquitin-like protein; HDAC, histone deacetylase

Regulation of protein synthesis is of fundamental importance to cells. It has a critical role in the control of gene expression, and consequently cell growth and proliferation. The importance of this control is supported by the fact that protein synthesis is frequently upregulated in tumor cells. The major point at which regulation occurs is the initiation stage. Initiation of translation involves the interaction of several proteins to form the eIF4F complex, the recognition of the mRNA by this complex, and the subsequent recruitment of the 40S ribosomal subunit to the mRNA. This results in the formation of the 48S complex that then scans the mRNA for the start codon, engages the methionyl-tRNA and eventually forms the mature 80S ribosome which is elongation-competent. Formation of the 48S complex is regulated by the availability of individual initiation factors and through specific protein-protein interactions. Both of these events can be regulated by post-translational modification by ubiquitin or UbIs (ubiquitin-like modifiers) such as SUMO or ISG15. We provide here a summary of translation initiation factors that are modified by ubiquitin or UbIs and, where they have been studied in detail, describe the role of these modifications and their effects on regulating protein synthesis.

protein (eIF4G), to form the eIF4F complex (eIF4E/eIF4A/eIF4G).¹⁻³ The eIF4G scaffold protein possesses domains that interact with eIF4E, eIF4A, eIF3 and the poly(A) binding protein, PABP.¹⁻⁴ PABP itself is regulated by interaction with other proteins; binding of Paip1 to PABP stimulates protein synthesis while interaction with Paip2 is inhibitory to translation.^{5,6} The activity of the eIF4F complex is regulated by a family of proteins, the eIF4E binding proteins (4E-BPs). Using a conserved motif, 4E-BPs compete with eIF4G for a common surface on eIF4E and inhibit eIF4F assembly. In mammalian cells, activation of the mechanistic target of rapamycin (mTORC1) leads to phosphorylation of 4E-BP1 in a hierarchical manner. This promotes protein synthesis by releasing eIF4E and enabling eIF4F complex assembly on the m⁷GTP cap of mRNA, mediating 40S ribosomal subunit binding by a bridging interaction between eIF4G and eIF3.¹⁻³

In most organisms there is more than one isoform of most of these translation initiation factors. For example, there are 3 isoforms of eIF4A, eIF4G and PABP.⁷⁻⁹ In some cases the functions of the isoforms are indistinguishable, in others there are indications that the different isoforms display mRNA-specific regulation.⁷⁻⁹ Further work will be required to uncover the full range of functions and specificities of these isoforms.

Introduction

Initiation of protein synthesis

Protein synthesis is of fundamental importance in cells and its regulation is crucial for the continued viability of organisms. The process comprises 3 stages: initiation, elongation and termination. Of these, initiation is generally considered to be one of the major regulatory steps of gene expression in mammalian cells. Initiation requires the function of a number of translation initiation factors (Fig. 1), several of which have key roles in cell survival and oncogenesis. These proteins modulate the binding of mRNA to the ribosome, a process facilitated by the assembly of the cap binding protein (eIF4E), a helicase (eIF4A) and a scaffold

Ubiquitin like proteins

Ubiquitin-like proteins (UbIs) comprise a family of structurally related proteins. The different members of the family share sequence similarities, and in particular the proteins contain a conserved β -grasp fold consisting of 5 β sheets and one α helix.¹⁰ Ubiquitin is a 76 amino acid protein and is the most highly conserved member of the Ubl family, with 96% identity between yeast and human ubiquitin. SUMO (small ubiquitin-like modifier) is less conserved between species and contains a longer, more variable N-terminal extension than ubiquitin being around 100–110 amino acids in total length.¹¹ ISG15, between 155–165 amino acids in length, contains 2 ubiquitin-like domains.¹² It was the first member of the family to be identified and, unlike ubiquitin and SUMO, is present only in vertebrates. The gene was so named because it was observed to be an interferon stimulated gene encoding a 15 kDa protein.¹³ Most members of the Ubl family are synthesized as precursor proteins that need to be processed to a mature form to reveal a di-glycine motif at the

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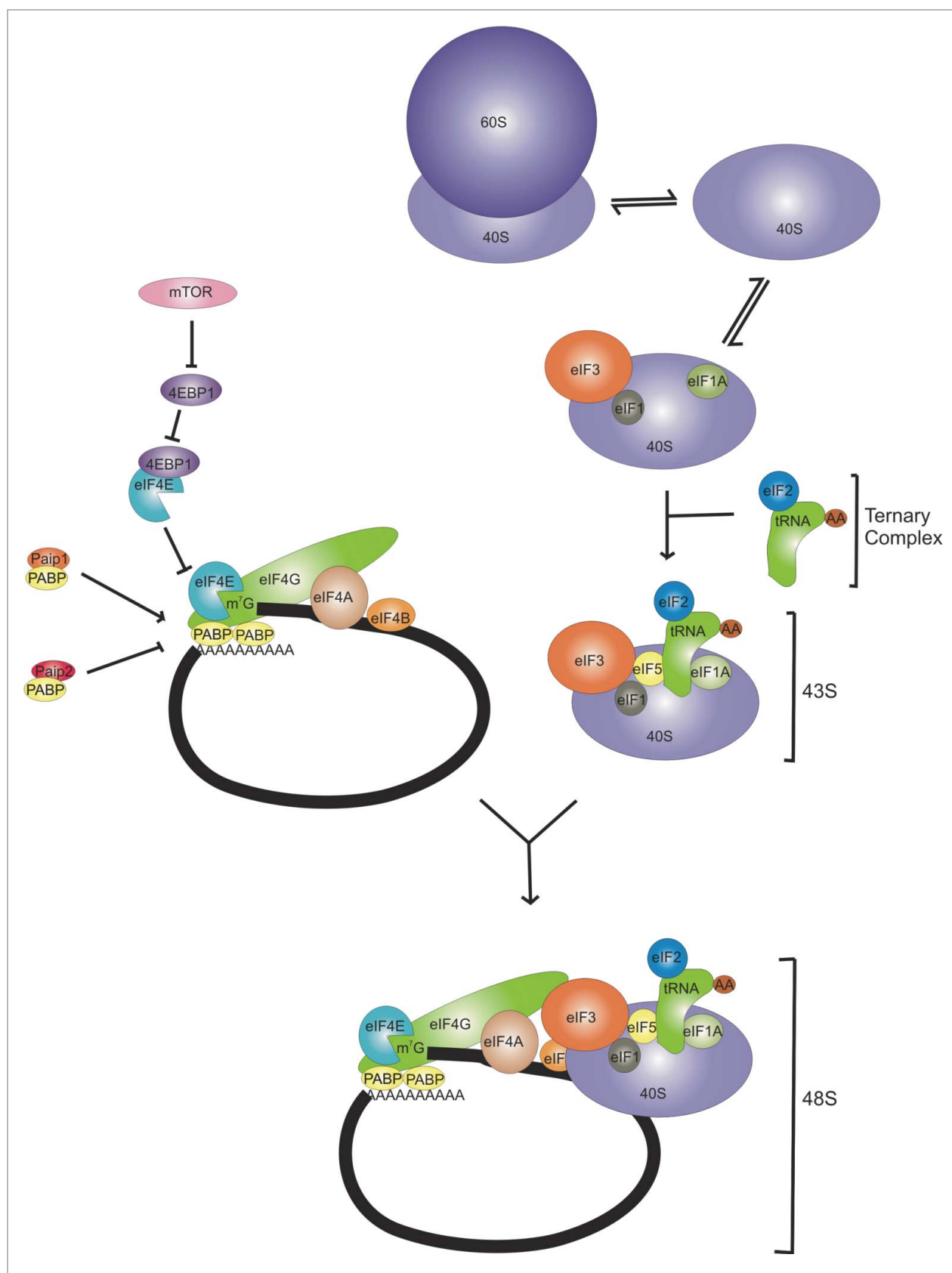


Figure 1. For figure legend, see page e959366-3.

C-terminus that is required for activation and subsequent conjugation of the Ubl to target proteins. The exception to this is ISG15 in fish and bovine species where the protein is synthesized in the mature form.¹⁴ Ubls are attached to one or more lysine residues in target proteins. There are no known consensus sequences for conjugation sites for ubiquitin and ISG15. However SUMO is frequently, although not always, attached to lysine residues present within the consensus sequence ψ KxE, where ψ = a hydrophobic amino acid and x is any amino acid.¹¹

Ubiquitylation

Ubiquitin can be covalently attached to lysine residues in target proteins as a monomer or in the form of chains. This occurs via the activity of a number of proteins, the E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) proteins (Fig. 2). In most organisms there is a single E1, around 40 E2s and hundreds of E3s (reviewed in^{15,16}). Ubiquitin is produced as a precursor protein that is processed to the mature form by one of a small number of specific ubiquitin proteases, to reveal a diglycine motif at the C-terminus. Ubiquitin is then activated in an ATP-dependent manner, by the formation of a thioester bond between the C-terminal glycine residue and a cysteine residue on the E1 activating enzyme. From here it is passed to an E2 ubiquitin conjugating enzyme, again, via the formation of a thioester bond between the C-terminal glycine residue and a cysteine residue. Attachment of ubiquitin requires one of a large number of E3 ubiquitin ligases, which in many cases interact directly with target proteins, but which in some instances interact with targets via an adaptor protein. In the main, the E3s provide the specificity for the modification. Ubiquitin chain formation occurs via lysine residues within ubiquitin itself, and also requires the activities of the E1, E2 and E3 enzymes. The most common linkages are via K11, K48 and K63.^{17,18} Ubiquitin can be removed from targets by the actions of deubiquitinating enzymes (DUBs). Ubiquitylation has 2 main roles: targeting of proteins for proteolysis and modification of protein function. The best studied role of ubiquitylation is its targeting of proteins for proteasome-mediated degradation. This involves the recognition of K11- and K48-linked ubiquitin chains by the 26S proteasome.¹⁹ However, there is a rapidly expanding literature on other roles for ubiquitylation. For example ubiquitylation of PCNA is required for the recruitment of an error-prone polymerase to undertake translesion DNA synthesis e.g.²⁰ while ubiquitylation of membrane proteins is required for endocytosis and ubiquitylation of PIN2 is required for vacuolar sorting (reviewed in²¹). In these cases the modification involves a single ubiquitin or K63-linked chains.

Sumoylation

The process of sumoylation is very similar to that of ubiquitylation, involving SUMO-specific E1 (SUMO activating enzyme), E2 (SUMO conjugating enzyme) and E3 (SUMO ligase) proteins.¹¹ There is a single E1 (a heterodimer), a single E2 (Ubc9) and to date around 12 E3s have been identified. Unlike ubiquitylation, an E3 is not always required for modification, as the E2 is in some cases sufficient, and can provide a degree of target specificity.²² Like ubiquitin, SUMO can be attached to proteins either as a monomer or in the form of poly-SUMO chains.¹¹ Sumoylation affects protein-protein interactions,^{23,24} protein activity²⁵ and protein localization.²⁶ In addition, SUMO chains interact with STUbLs (SUMO-targeted ubiquitin ligases) that bring about ubiquitylation of the target protein and associated SUMO chains, resulting in proteasome-mediated proteolysis.²⁷

ISGylation

ISG15 is conjugated to target proteins in a manner similar to that of ubiquitin and SUMO.²⁸ ISG15 expression and modification (ISGylation) are activated by Type I interferon (IFN), which is one of a number of critical cytokines in the innate immune response. As is the case for ubiquitin and SUMO, there are proteases that are specific for processing ISG15 and deconjugating it from target proteins (e.g., USP43,²⁹) and a specific E1 enzyme for ISG15.²⁹ However, some of the E2s (e.g., UbcH8) and E3s (e.g., Efp—the partner of UbcH8, and HHARI—the human homolog of *Drosophila* ariadne) involved in ISGylation also appear to be involved in ubiquitylation.^{30,31}

Identification of Ubl Attachment Sites and the Roles of Modification

Early methods for the identification of modified sites involved site-directed mutagenesis of individual lysine residues in target proteins, followed by analysis in vitro or in vivo to determine whether modification still occurred. While this has been successful in some cases (e.g.,³²) in many cases it has been problematic since other lysine residues are frequently used instead of the normal sites in the mutant proteins. More recently, mass spectrometry has been used successfully for site identification (e.g.,³³). This involves the cleavage of modified proteins by trypsin or other suitable protease to release peptides from the target. This method is facilitated by having a protease cleavage site close to the C-terminal diglycine motif attached to the target, so that only a few extra amino acids remain attached to the modified site. Modification sites are thus detected by the identification of peptides that are increased in Mr by an amount dependent on the position of the cleavage site within the Ubl.

Figure 1. (See previous page). Formation of the 48S preinitiation complex. eIF1, 1A and 3 interact with the 40S ribosomal subunit. This then interacts with eIF5 and the ternary complex (eIF2-GTP-Met-tRNA) to form the 43S complex. In parallel, eIF4E and eIF4A are recruited by eIF4G to form the eIF4F complex. The availability of eIF4E is controlled by 4E-BP1, which in turn is regulated by phosphorylation by mTOR. The eIF4F complex binds to the cap on mRNA along with Poly(A)-binding protein (PABP) and eIF4B. PABP is regulated via interactions with 2 PABP proteins, PAIP1 and PAIP2. The 43S complex then binds close to the cap from where it can scan the mRNA for the start codon.

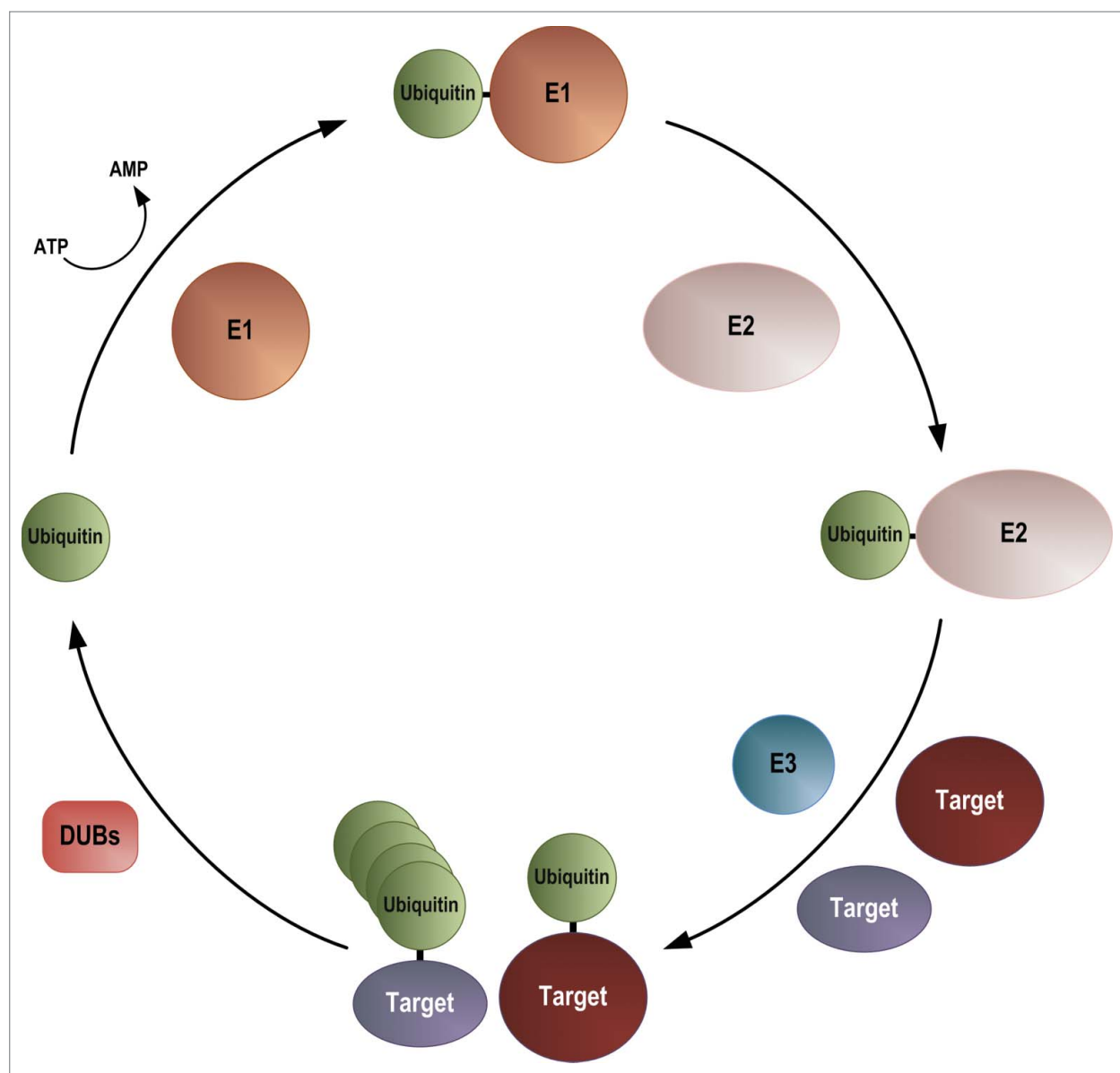


Figure 2. Ubiquitylation pathway. E1 = Ubiquitin activating enzyme, E2 = ubiquitin conjugating enzyme, E3 = ubiquitin ligase, DUB = deubiquitylating enzyme. Ubiquitin is activated by the formation of a ubiquitin-adenylate before forming a thioester bond with a cysteine residue in the E1 ubiquitin activating enzyme. Ubiquitin is passed to an E2 ubiquitin conjugating enzyme, again forming a thioester bond. Target proteins are recognized by E3 ubiquitin ligases, either directly or via an adaptor, and ubiquitin is attached via the formation of an ϵ -amino bond. Ubiquitin can be attached to target proteins either as a monomer, or in the form of ubiquitin chains. Ubiquitin can be removed from target proteins by the action of one of a number of DUBs.

Analysis of the role of the modifications is hampered by the fact that frequently, only low levels of modified forms are observed in cells. The reason for this could be that the modifications are transient, are labile, or as in the case of poly-ubiquitylation and poly-sumoylation, are targeting the protein for proteasome-mediated destruction. It is also possible that modification may be confined to target molecules in a particular cellular location. Additionally, it is proposed that this form of post-translational modification is not like modifications such as phosphorylation—i.e., an on/off switch. For example, in the case of SUMO, it is proposed that in some cases modification results in

a change in conformation of the target protein that is maintained even after desumoylation occurs. Thus analysis of the roles of these modifications has lagged behind analysis of the function of other types of modifications.

Identification of the roles of the modifications has been undertaken, in the main using *in vitro* assays to look at relative binding abilities of wild type and unsumoylatable mutant proteins for their binding partners e.g.,³² or by introduction of mutant coding sequences into cells to determine the effect of inability to modify a particular protein. This is relatively straightforward in yeast where a mutant copy can be integrated in the genome as

the sole copy of the coding sequence e.g.,³⁴ In mammalian cells, the mutant sequence can be introduced by transfection, but is dependent on having cells where the gene has been knocked out or where siRNA depletion is efficient. Depletion of the any of the enzymes in the conjugation pathway would be likely to affect multiple targets and would not be appropriate.

Role of Modification by Ubiquitin or Ubls in Translation Initiation Factors

A series of recent proteomic screens have identified numerous translation initiation factors that are modified by either ubiquitin or SUMO, or in many cases, by both (Table 1). Additionally, some of the screens have identified the lysine residues required for the modification. Early studies involved the overexpression of the modifier, but recently more refined methods using diGly capture techniques have been used to identify sites when the

modifier is expressed at endogenous levels e.g.^{35,36} These studies use mass spectrometry to identify diGly-modified peptides obtained by trypsin digestion of cellular proteins. A list of modified sites can be found at PhosphoSitePlus³⁷ (<http://www.phosphosite.org/home>). In many cases, individual lysine residues are identified as a single 'hit', making them less likely target sites than lysine residues that are highly represented, as for example is observed in eIF4A and eIF4G proteins.

More detailed studies on the role of modification of a number of the individual proteins by ubiquitin, SUMO and in one case, ISG15 have also been reported. We summarize here what is known about the roles of these post-translational modifications and how they might affect translation rates in mammalian cells.

eIF4E

Regulation of eIF4E levels is important for normal cell growth, as disruption of its expression or its over-production leads to aberrant cell growth or oncogenesis.³⁸ Additionally,

Table 1. Proteins identified in proteomic screens as being modified by ubiquitin or SUMO

Initiation factor	Ubiquitin	SUMO	Reference
eIF1A	Hs Ubiquitin*Mm Ubiquitin	Rn SUMO-3	35,36,58,59
eIF2A	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-2*At SUMO	35,55,59,80
eIF2α	Hs Ubiquitin*Mm Ubiquitin	Dm SUMO	36,56,59
eIF2B-β	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-1/2	36,59,81
eIF2β	Hs Ubiquitin*Mm Ubiquitin	At SUMO*Sc SUMO-1	36,59,80,82
eIF2 subunit 1	Hs Ubiquitin*Mm Ubiquitin	Rn SUMO-3	35,58,59
eIF2γ	Hs Ubiquitin*Mm Ubiquitin	Dm SUMO*Hs SUMO-1*Hs SUMO-2/3*Sc SUMO	35,36,56,57,59,82,83, *
eIF5B*	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-2*Hs SUMO-1*	55,59,61 (A)
eIF3A	Hs Ubiquitin*Mm Ubiquitin*Rn Ubiquitin	Hs SUMO-2*Hs SUMO-1	36,55,59,61 (B)*
eIF3B	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-2	35,55,59
eIF3C	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO 1/2	35,59,81
eIF3D	Hs Ubiquitin*Mm Ubiquitin	Rn SUMO-3	35,58,59
eIF3E	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-1/2	35,59,81
eIF3F	Hs Ubiquitin*Mm Ubiquitin		59,74 (C)
eIF3G	Hs Ubiquitin*Mm Ubiquitin		35,36,59
eIF3H	Hs Ubiquitin*Mm Ubiquitin*Rn Ubiquitin		35,36,59 (D)*
eIF3I	Hs Ubiquitin	Sc SUMO*Hs SUMO-1/2	35,36,59,60,82–84
eIF3J	Hs Ubiquitin*Mm Ubiquitin		36,59
eIF3K	Hs Ubiquitin*Mm Ubiquitin		35,36*
eIF3L	Hs Ubiquitin*Mm Ubiquitin		35,59
eIF3M	Hs Ubiquitin*Mm Ubiquitin		35,59,83
eIF3X		Hs SUMO-1	55
eIF4A1	Hs Ubiquitin*Mm Ubiquitin*Rn Ubiquitin	Dm SUMO*Rn SUMO-3*Hs SUMO-1/2*At SUMO	35,36,55–62 (D)
eIF4A2	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-1	35,59,61
eIF4E	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-1	36,46,59
eIF4G1	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-1/2	36,57,59,61
eIF4GII	Hs Ubiquitin		35
eIF4GIII	Hs Ubiquitin*Mm Ubiquitin		35,36
eIF5A	Hs Ubiquitin*Mm Ubiquitin*Rn Ubiquitin	Hs SUMO-1/2	35,59,83 (D)
PABP1	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-2*Sc SUMO	35,55,57,59,85
PABP4	Hs Ubiquitin*Mm Ubiquitin	Hs SUMO-2	35,55,59

Hs: human, Rn: rat, Mm: mouse, Sc: *S. cerevisiae*, At: *Arabidopsis*. (A) (2010) CST Curation Set: 9913; Year: 2010; SILAC: N; Biosample/Treatment: AMO-1(cell line)/Velcade; Disease: -; Specificity of Antibody Used to Purify Peptides prior to MS2: anti-UbK Antibody Used to Purify Peptides prior to MS2: Ubiquitin (D4A7A10) XP(R) Rabbit mAb Cat#: 3925, PTMScan(R) Ubiquitin Branch Motif (K-e-GG) Immunoaffinity Beads Cat#: 1990. (B) (2008) CST Curation Set: 3970; Year: 2008; SILAC: N; Biosample/Treatment: brain(tissue)/untreated; Disease: -; Specificity of Antibody Used to Purify Peptides prior to MS2: anti-UbK. (C) (2009) CST Curation Set: 8668; Year: 2009; SILAC: N; Biosample/Treatment: RPMI-8266(cell line)/Velcade; Disease: -; Specificity of Antibody Used to Purify Peptides prior to MS2: anti-UbK Antibody Used to Purify Peptides prior to MS2: Ubiquitin (D4A7A10) XP(R) Rabbit mAb Cat#: 3925, PTMScan(R) Ubiquitin Branch Motif (K-e-GG) Immunoaffinity Beads Cat#: 1990. (D) (2007) CST Curation Set: 3578; Year: 2007; SILAC: N; Biosample/Treatment: brain(tissue)/ischemia and Reperfusion; Disease: -; Specificity of Antibody Used to Purify Peptides prior to MS2: anti-UbK.

eIF4E protein levels increase during differentiation e.g.,³⁹ eIF4E is both mono- and poly-ubiquitylated^{40,41} and this has been demonstrated to occur mainly on K159.⁴⁰ This modification is enhanced by the E3 ubiquitin ligase, Chip (carboxy terminus of Hsp-70 interacting protein) which is known to have a role in regulating protein quality control.⁴² A mutant form of eIF4E that is unable to interact with eIF4G or 4E-BP1 is more highly ubiquitylated than wild type eIF4E. This results in increased degradation by the proteasome of the mutant form, consistent with a role for ubiquitylation of eIF4E in a quality control process, removing inactive forms of the protein from the cell.⁴⁰ A role for ubiquitylation in quality control is supported by a number of observations. First, that binding of eIF4E to 4E-BP1 (eIF4E binding protein that is also regulated by ubiquitylation—see below) suppresses ubiquitylation and degradation and that only non-ubiquitylated eIF4E binds eIF4G. Second, overexpression of 4E-BP1 prevents ubiquitin-mediated degradation of eIF4E. Third, heat shock (45°C 10 min, conditions that would result in a degree of protein misfolding) also induces ubiquitylation of eIF4E, as does exposure to another form of stress, cadmium chloride.⁴¹

While poly-ubiquitylation clearly has a role in targeted destruction of eIF4E, little work has been performed to determine whether there is a different role for mono-ubiquitylation in regulating levels or subcellular localization of eIF4E. In contrast, the biological significance of eIF4E phosphorylation and its effect on translation have been studied over many years; however, the role of phosphorylation in modulating the activity of the protein is still not completely understood, although enhanced levels of eIF4E phosphorylation are associated with a number of human tumors.^{43,44} Biophysical studies have suggested that phosphorylation of eIF4E decreases its affinity for the mRNA cap of mRNA, possibly allowing rapid recycling of eIF4E between competing mRNAs.⁴⁵ However, it has also been suggested that phosphorylation of S209 causes a retractable salt bridge to form with K159 (the ubiquitylation site) which leads to increased binding of capped mRNA.⁴⁰ Mutation of K159 to alanine but not arginine, reduces association with cap analogs, indicating that a positive charge is required at this position. Despite the fact that the K159R mutant cannot be ubiquitylated, it has been proposed that mono-ubiquitylation may stabilize the distance between S209 and K159, or that ubiquitin itself may form part of the bridge between S209 and K159.⁴⁰

eIF4E is also modified by SUMO,^{32,46} in a process that is promoted by HDAC2 (histone deacetylase 2).⁴⁶ Sumoylation occurs on several lysine residues, namely K36, 49, 162, 206 and 212. Interestingly, unlike what has been observed with a number of other proteins, such as IκBα and PCNA,^{34,47} sumoylation and ubiquitylation of eIF4E do not occur on the same lysine residues. Sumoylation of eIF4E is dependent on phosphorylation, but the reverse is not true: inability to sumoylate eIF4E does not affect its ability to be phosphorylated.³² Sumoylation results in the induction of translation of a subset of mRNAs required for cell proliferation and apoptosis. A mutant form of eIF4E that cannot be sumoylated is still able to bind m⁷GTP, indicating that cap-binding is unaffected. However, compared with wild type

protein, the mutant form binds significantly better to 4E-BP1 than it does to eIF4G, and is unable to form stable eIF4F complexes. It has been suggested that sumoylation induces a conformational change in eIF4E producing a change in interaction surfaces resulting in release from 4E-BP1 and promoting interaction with eIF4G. The inability of the mutant protein to be sumoylated results in an increase in the amount of eIF4E interacting with 4E-BP1.³² While overexpression of wild type eIF4E in NIH-3T3 cells results in increased expression of eIF4E-regulated genes, this is not observed when unsumoylatable eIF4E is overexpressed.³² At this time is unclear whether sumoylation of eIF4E has any effect of global rates of translation or rates of export of specific mRNAs from the nucleus.

4EHP

4EHP, also known as eIF4E2, binds to the m⁷GTP cap in a manner similar to that of eIF4E. However, unlike eIF4E, it does not bind eIF4G and therefore does not allow ribosome recruitment. It thus competes with eIF4E for the mRNA and prevents translation.⁴⁸ It is targeted for ubiquitylation⁴⁹ and interestingly, also for modification with another Ubl, ISG15.⁵⁰ Curiously, the E3 ligase HHARI, which has recently been shown to be a marker of cellular proliferation,⁵¹ stimulates both ubiquitylation and ISGylation of 4EHP.^{49,50} Proteomic studies have identified K239 as a ubiquitylation site, but this has not been verified in a detailed study. In contrast, ISGylation, which occurs on K134 and K222, has been analyzed in some detail.⁵⁰ Binding studies indicate that ISGylated 4EHP has a higher affinity for m⁷GTP than the unmodified form. It has been proposed that this modification is used by cells to inhibit translation of specific mRNAs in innate immune responses. Interestingly, despite its similarity to 4EHP, eIF4E is not ISGylated.

4E-BP Family

The eIF4E binding proteins (4E-BPs) are key regulators of protein synthesis.¹⁻³ As their name suggests, they function by interacting with eIF4E. This inhibits eIF4E function by preventing it from interacting with eIF4G to form the mature eIF4F complex. The 4E-BP proteins are phosphorylated following activation of mTORC1, in response to changes in growth conditions, and interaction of eIF4E with 4E-BP1 and -2 occurs with the hypophosphorylated form.¹⁻⁵ A key factor in the regulation of translation initiation is that the relative levels of eIF4E and 4E-BP1 and -2 are highly controlled.⁵² The hypophosphorylated form, but not the hyperphosphorylated form, of 4E-BP1 is unstable if not bound to eIF4E. Under these conditions, 4E-BP1 is ubiquitylated and targeted for proteasome-mediated proteolysis.^{52,53} The role of ubiquitylation was identified following some rather unexpected results obtained when knockdown of eIF4E using shRNA was demonstrated to have no effect on protein synthesis.⁵² This was subsequently shown to be due to concomitant degradation of 4E-BP1, which resulted in the release of eIF4E molecules to compensate for the loss brought about by the reduced expression. K57, a lysine residue conserved between all 3 4E-BPs, was identified by the Sonenberg lab as the ubiquitylation site in 4E-BP1,⁵² and a screen of an siRNA library identified the

KLHL25-CUL3 as the E3 ubiquitin ligase responsible for 4E-BP1 degradation. Knockdown of KLHL25 resulted in a decrease in translation, consistent with it having a role in controlling levels of 4E-BP1.⁵²

Proteasome activity (presumed to be a result of poly-ubiquitylation) has also been demonstrated to be required for the formation of a truncated form of 4E-BP1 (tr4E-BP) in murine erythroleukemia (MEL) cells containing activated p53.⁵⁴ This truncated form is 3 kDa smaller than full-length protein, is unphosphorylated and relatively stable. It also binds to eIF4E in preference to the full-length protein. It has been proposed that the production of this p53-induced form may be contributing to the ability of p53 to regulate apoptosis and malignancy.

eIF4A

Two isoforms of eIF4A have been identified in proteomic screens as being modified by ubiquitin and SUMO.^{35,36,55-62} In contrast to what is observed with some of the other initiation factors, modified peptides from both eIF4A1 and eIF4A2 are highly abundant in the proteomic screens designed to identify ubiquitylation sites, implying that modification is likely to have a key role(s) in the regulation of the function of these 2 proteins. In the ubiquitin screens, most of the modified sites identified in the human eIF4A proteins were also observed in the mouse proteins, suggesting that they are likely to be true 'hits' and not false positives. Interestingly, eIF4A2 (but not eIF4A1) and translational repression have both been shown to be essential for miRNA-mediated gene regulation.⁶³ However, the post-translational modification of these proteins by ubiquitin or UbIs has not been analyzed in detail and to date there are no reports on whether it affects the activity of the eIF4A protein or miRNA-mediated translational control.

In a role unrelated to its function in translation, ubiquitylation of *Drosophila* eIF4A has been shown to be linked with Decapentaplegic (Dpp) signaling.⁶⁴ Additionally, rice DRM2 (required for RNA-directed DNA methylation) interacts with eIF4A via its ubiquitin associated (UBA) domain, (although whether this occurs with a ubiquitylated form has not been analyzed).⁶⁵

eIF4G

There are 3 isoforms of the scaffold protein, eIF4G, eIF4GI-III. As observed with eIF4A, diGly-modified peptides from these proteins are abundant in proteomic screens designed to identify ubiquitylation sites,^{35,36,57,59,61} and again most are observed in both the human and mouse proteins. In eIF4GI these sites (6 in total, 4 common to both human and mouse) map to lysine residues occurring between amino acids 593–925 which map close to, or in the region of, the eIF4E and eIF4A/3 binding sites. The abundance of these modified tryptic fragments and their position in the protein suggests that this post-translational modification is likely to be important for regulating the functions of these proteins, possibly by affecting the interaction of eIF4G with other members of the eIF4F complex. Again, these modifications have not been analyzed in detail and to date there are no reports on whether they affect the activity of eIF4GI. In addition to this

modification by ubiquitin, eIF4GI has been shown to be sumoylated in both fission yeast and human cells.⁶⁶ Sumoylation of *S. pombe* eIF4G is increased following exposure of cells to 1 M KCl or arsenite, conditions which result in the formation of stress granules. In vitro sumoylation studies have identified 2 sumoylation sites in mammalian eIF4GI, K1368 and K1588, residing in the eIF3/4A binding site and the Mnk-binding domain, respectively. (Mnks (MAP kinase-interacting kinases) are kinases which bind to the C-terminus of eIF4G and phosphorylate eIF4E which is bound to the N-terminus of eIF4G.⁶⁷) These data suggest that sumoylation may be affecting interactions of eIF4GI with associated proteins, e.g., eIF4E, and possibly the assembly of eIF4G into stress granules.

Paip2

Poly(A)-binding protein (PABP) is regulated through the interaction with 2 proteins, Paip1 and Paip2.^{5,6} Paip1, which also interacts with eIF3g, is eIF4G-like and is stimulatory for translation, while Paip2 represses PABP function by decreasing the affinity of PABP for polyadenylated mRNA, thus inhibiting translation. Paip1 and Paip2 both have 2 domains, PAM1 and PAM2 which interact with PABP. This interaction occurs through RRM-1 and PABC domains, respectively.⁶⁸ Additionally, PAM2 is capable of interacting with EDD (a member of the HECT domain family of E3 ubiquitin ligases) which also contains a PABC domain.⁶⁹ In cells where levels of PABP are depleted, Paip2A is ubiquitylated in an EDD-dependent manner prior to proteasome-mediated degradation.⁷⁰ Interestingly, the affinity of the PAM2 domain of Paip2 for the PABC domain of PABP is greater than that of the affinity for the PABC of EDD. Thus, it is proposed that interaction of PABP with Paip2 competes with EDD for interaction with PAM2 on Paip2, and that this normally prevents ubiquitylation of Paip2.⁷⁰ However, in apparently contradictory work, it has been demonstrated that during human cytomegalovirus infection PABP levels rise concomitantly with the levels of both Paip2 and EDD1. The reason for this is not known, but it has been proposed that it may provide cells with a process to allow rapid changes in protein levels if necessary.⁷¹ Paip2B is also polyubiquitylated, although at a somewhat lower level than Paip2, and is hence more stable.⁷²

eIF3

Proteomic studies have identified many of the eIF3 subunits as targets for ubiquitylation and/or sumoylation. However, independent of these studies, eIF3f is the only eIF3 subunit where the function of these modifications has been studied in any detail. eIF3f is a non-core subunit of the eIF3 complex. It can act both as a repressor and as an enhancer of translation (reviewed in⁷³). Its role as a translational enhancer came to light in a study on muscle atrophy.⁷⁴ Here, eIF3f is ubiquitylated by the MAFbnx/Atrogin1 protein which is a muscle-specific F-box protein ubiquitin E3 ligase.⁷⁵ This E3 is upregulated and essential for accelerated muscle protein loss in a number of disorders.⁷⁶ Ubiquitylation of eIF3f occurs on multiple (6) lysines in the C-terminus⁷⁴ and results in its ubiquitin-mediated proteolysis in myotubes undergoing atrophy. Under these conditions both

MAFbnx and eIF3f are detected in the nucleus.⁷⁵ It has been proposed that this ubiquitylation may be associated with the rapid downregulation of certain proteins during muscle atrophy. eIF3f also interacts with the ubiquitin E3 ligase TRC8 to inhibit protein synthesis. The mechanism by which this occurs is unknown, but it has been proposed that TRC8 targets an eIF3 subunit for ubiquitylation.⁷⁷ Unrelated to its role in translation, eIF3f can act as a deubiquitylating enzyme (DUB). In this capacity it is capable of deubiquitylating, and thus contributing to the activation of, the Notch signaling receptor in *Drosophila*.⁷⁸

Interestingly, recent work has shown that eIF3e is involved in eIF4E phosphorylation; Mnk1 binding to eIF4F is dependent on eIF3e, and eIF3e is sufficient to promote Mnk1-binding to eIF4G.⁷⁹ As eIF3e is modified by both ubiquitylation and sumoylation, it would be interesting to know if these modifications of eIF3e also have a role in controlling eIF4E phosphorylation.

Summary

In conclusion, despite the fact that numerous translation initiation factors have been shown to be ubiquitylated and/or sumoylated in proteomic screens, relatively little is known about the

effects of the modifications on the functions of individual proteins. In part this is due to the transient nature of these modifications, e.g., in many cases of sumoylation, less than 5% of a particular protein is modified at any one time, and the sumoylated species appear to be very labile in certain organisms due to highly active SUMO-specific proteases. Additionally, since ubiquitylation targets proteins for destruction, analysis of ubiquitylated proteins, other than in the presence of a proteasome inhibitor, is difficult.

The recent use of proteomic screens to identify modified proteins and the modified site(s) suggests that there are many more cases where post-translational modification by ubiquitin or Ubls is likely to affect translation initiation factors. For example, sumoylation of eIF4A1/2 might have a role in regulating both the unwinding of mRNA secondary structure and the ability of eIF4A2 to mediate miRNA-dependent gene expression in mammalian cells. Further work on these modifications is required to fully elucidate their effect on individual proteins and on translational control of gene expression as a whole.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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